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ELECTRON MICROSCOPY OF THE PANCREATIC ISLETS[1]

SERGIO A. BENCOSME[2] AND DANIEL C. PEASE

Department of Anatomy, School of Medicine, University of California Los Angeles 24, Veterans Administration Center, Los Angeles 25, California, and the Department of Pathology, Queen's University, Kingston, Ontario, Canada

ABSTRACT

Pancreatic islets have been studied by the electron microscopy of ultrathin sections in mature guinea pigs and cats.

Alpha cells contain specific granules which are notably dense after fixation with osmium tetroxide. Their substance is homogeneous. They are enclosed within a vacuolar membrane. Other organelles such as the Golgi apparatus, endoplasmic reticulum, RNA granules and mitochondria are present inconspicuously, and without particular specialization.

Beta cells of the cat and guinea pig differ in important respects. In the cat the specific granules contain an angular crystalloid element. The homologous structure in the guinea pig is spherical. These granules are enclosed in membranous envelopes. The density of the granules is substantially less than those of alpha cells.

Beta cells also differ from alpha cells in having a richer development of the endoplasmic reticular system, Golgi apparatus and RNA granules. The guinea pig beta cells also show a marked polarization of organelles. Thus, it seems clear that the two cell types represent fundamentally different cells, and it is significant that in the cat, where identification is simple, no granules of the alpha cell were ever found interspersed, within a single cell, with granules of the beta cell type.

Delta cells were studied, particularly in the cat. A nongranular cell is to be found having a cytoplasm suggesting a vacuolar compartmentalization. An apparently similar cell, but containing specific granules of a unique type, is also observed. The former may be regarded as the typical delta cell, the latter as a granulated variant.

Islet cells are separated from their blood supply by the capillary wall, a layer of connective tissue, a parenchymal basement membrane and sometimes also by a subcellular space. Autonomic nerve bundles are seen in the pericapillary connective tissue. Capillary walls are frequently fenestrated, the

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attenuated endothelial sheet being interrupted by a regularly spaced system of pores having a diameter of approximately 0.05 micron. The only continuous barrier between plasma and connective tissue fluid would thus seem to be the thin basement membrane underlying the endothelium.

AT THE present time there is no detailed published account concerned with the electron microscopy of the islets of Langerhans. The most important work to date is that of Lacy (1 and 2) who has presented an abstract and a short paper concerned with cell identification. Additionally, Hartroft (3) has published several micrographs accredited to Doctor Lacy. Dalton (4), Robertson (5), and Palade (6) have made minor references to the islet cells in papers concerned mainly with the exocrine tissues of the pancreas. This being the extent of knowledge it is, therefore, appropriate to describe the islets in some detail.

MATERIALS AND METHODS

Adult male cats and guinea pigs were used in these experiments. Laparotomy was performed in all cases during anesthesia induced by intraperitoneal injection of sodium amytal. In guinea pigs, single pancreatic islets were identified with a stereoscopic dissecting microscope and their surfaces partially denuded before fixation. Individual islets were then flushed for 5 to 10 minutes in the living animal with chilled buffered osmium tetroxide fixative (7). Single isolated islets were removed with iridectomy scissors and immersed in the fixing fluid for another 20 to 30 minutes. Effort was made to avoid seriously disturbing the islet circulation until the final removal of the tissues.

The islets of the cat could not be visualized in the living animal. Therefore, the pancreas was almost completely transected under anesthesia and the cut surface flushed with chilled osmic acid for 5 to 10 minutes. Subsequently small fragments of tissue were removed from near the center of the pancreas where islets are known to be largest and most numerous. These samples were given a further 20 to 30 minute fixation before dehydration.

Fixed tissues were dehydrated within a one-half hour period in increasing concentrations of ethyl alcohol. They were infiltrated in n-butyl methacrylate and cut with a Porter-Blum microtome equipped with a glass knife.

From the blocks used for electron microscopy, sections 0.5 to 2μ in thickness were stained with a modified Masson's trichrome stain and Gomori's chrome alum hematoxylin (8). Gomori's aldehyde-fuchsin stain was also used (9). In this manner, cytological correlation of light and electron microscopy was possible.

OBSERVATIONS AND CONCLUSIONS

The three principal cell types to be found in pancreatic islets are more easily distinguished in the cat than in the guinea pig. These are readily seen in Figure 1. The cells with extremely dense spherical granules can be identi-

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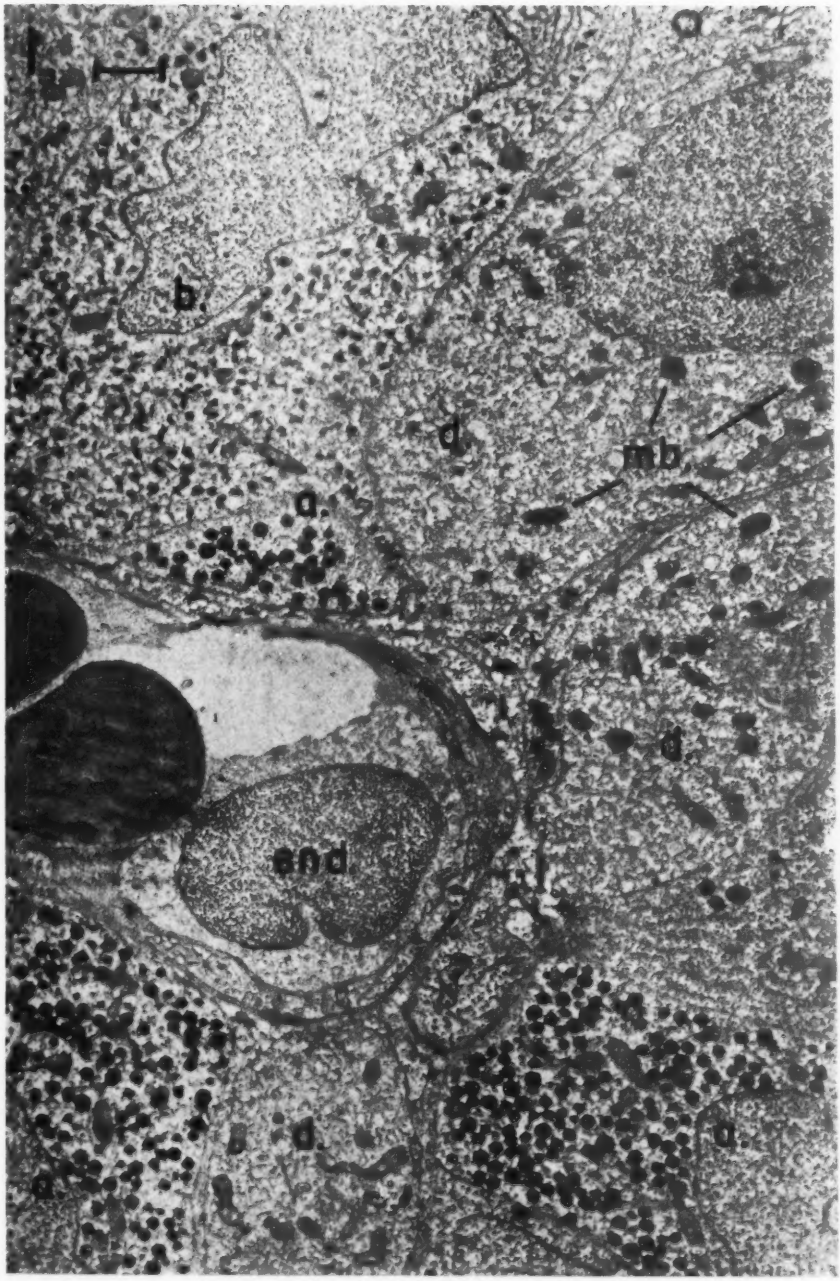
FIG. 1. Cat. Relatively low-power view of a pancreatic islet showing portions of three alpha cells (*a*) with dense, spherical granules. One visible beta cell (*b*) has granules of a different type. Portions of three agranular delta cells (*d*) are also visible. Microbodies (mb) as described in the text are visible in the delta cells. Micron marks accompany this and all subsequent figures.


a
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d

fied as alpha cells because of their sparse distribution and their tendency to aggregate in small clusters. By far the commonest cell type is the beta cell, and its granules appear elongate and with decidedly less density than those of alpha cells. The third cell type, the delta cell, is quite rare and ordinarily lacks granules, although the cytoplasm is "vacuolated."

In the guinea pig, the granules of both the alpha and beta cells are spherical and of nearly the same size (Fig. 7). Although the granules differ in density, a single isolated cell may be confused easily.

Alpha Cells. There is no notable species difference between the cat and the guinea pig insofar as the alpha cells are concerned (compare Figs. 4 and 7). The material of the alpha cell granules has great density and ends abruptly with a sharp border. The granules of the alpha cells vary in size. The largest ones observed in the cat approach 0.3 μ in diameter, and in the guinea pig, 0.2 μ. Their substance appears essentially homogeneous (Fig. 3). Variations in apparent density from one granule to another may be observed. These variations should be interpreted cautiously as being due to differences in material concentration because they may be dependent upon variable section thickness. It is characteristic of these granules that each is enclosed in a membranous envelope and separated from the latter by a narrow space approximately 0.02 μ in width.

The granules of the alpha cells are fairly uniformly distributed throughout the cell except in the cytocentrum, which is occupied by the Golgi apparatus and most of the membranes of the endoplasmic reticulum. Mitochondria appear to be most numerous in the cytocentrum, although many are present throughout the rest of the cytoplasm. Numerous small granules, presumably of the RNA type (10), are distributed throughout the cytoplasm, and supported in an amorphous matrix of low density (Fig. 7).

Alpha cells always appear fairly well packed with their specific granules. In the cat, where the distinction between granules of the alpha and beta cells was easy to determine, a particular effort was made to see whether there was ever any mixing of the granules within a single cell. No instance of this was observed, so we feel justified in believing that the alpha and beta cells are each uniquely specialized and do not intergrade one with the other.

Beta Cells. The granules of the beta cells of the cat have embedded within their substance a solid dense component. When viewed in one plane this appears as a lozenge (Fig. 2, granules indicated by *x*), in the other plane as an ellipse or circle (Fig. 2, granules indicated by *y*). Rarely, multiple

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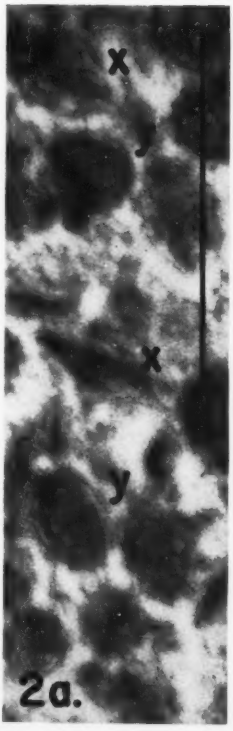
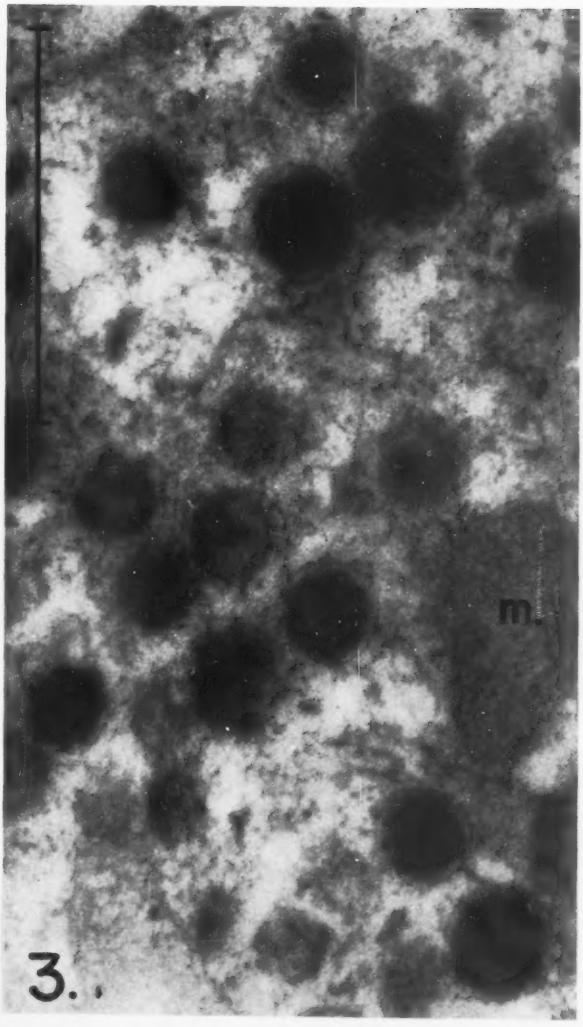
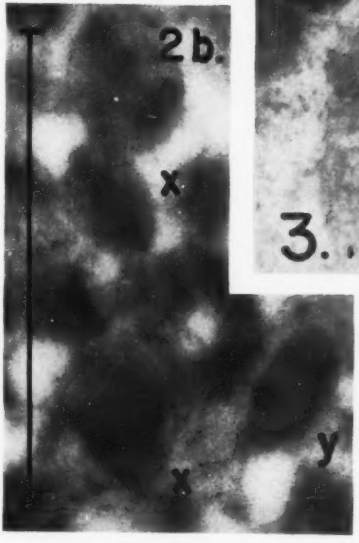
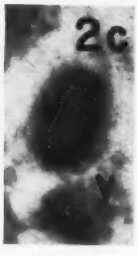
FIGS. 2a–e. Cat. Granules of the beta cell at high magnification showing an outer envelope surrounding an amorphous matrix and a crystalloid inclusion. When viewed in one plane (*X*), the inclusions appear sharply angular and lozenge shaped. When viewed in the other plane (*Y*), the inclusions have an elliptical profile.

FIG. 3 Cat. Granules of the alpha cell at high magnification showing the peripheral envelope. The inclusions appear to be homogeneous in character.


x
x
y
2a.


m.
3.


2b.
x
x
y


2c
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discs of this sort may be found within a single granule (Fig. 2d, e). The sharply angular character of these bodies suggests a semicrystalline organization. A somewhat comparable inclusion is reported by Lacy (2) in the dog beta cell.

The granules of the beta cells are enveloped by membranes much like those investing the granules of alpha cells (Fig. 2). The envelope encloses an unorganized matrix of substantial density as well as the crystalloid material. These membranes are without specific affinity for RNA granules, and thus are not regarded as a part of the endoplasmic reticular system. The latter system is quite well developed in beta cells as described below, and there is no possibility of confusion.

In the guinea pig, the granules of the beta cells are spherical and enclosed in a membrane that does not make contact with the surface of the granule. In this species, granules of the beta and alpha cells are distinguishable mainly on the basis of density (Fig. 7). In both the guinea pig and the cat, the granules of the beta cells lack the extreme density that characterizes those of the alpha cells (Figs. 1, 7). Lacy (2) also reports spherical granules of moderate density in the rat and rabbit.

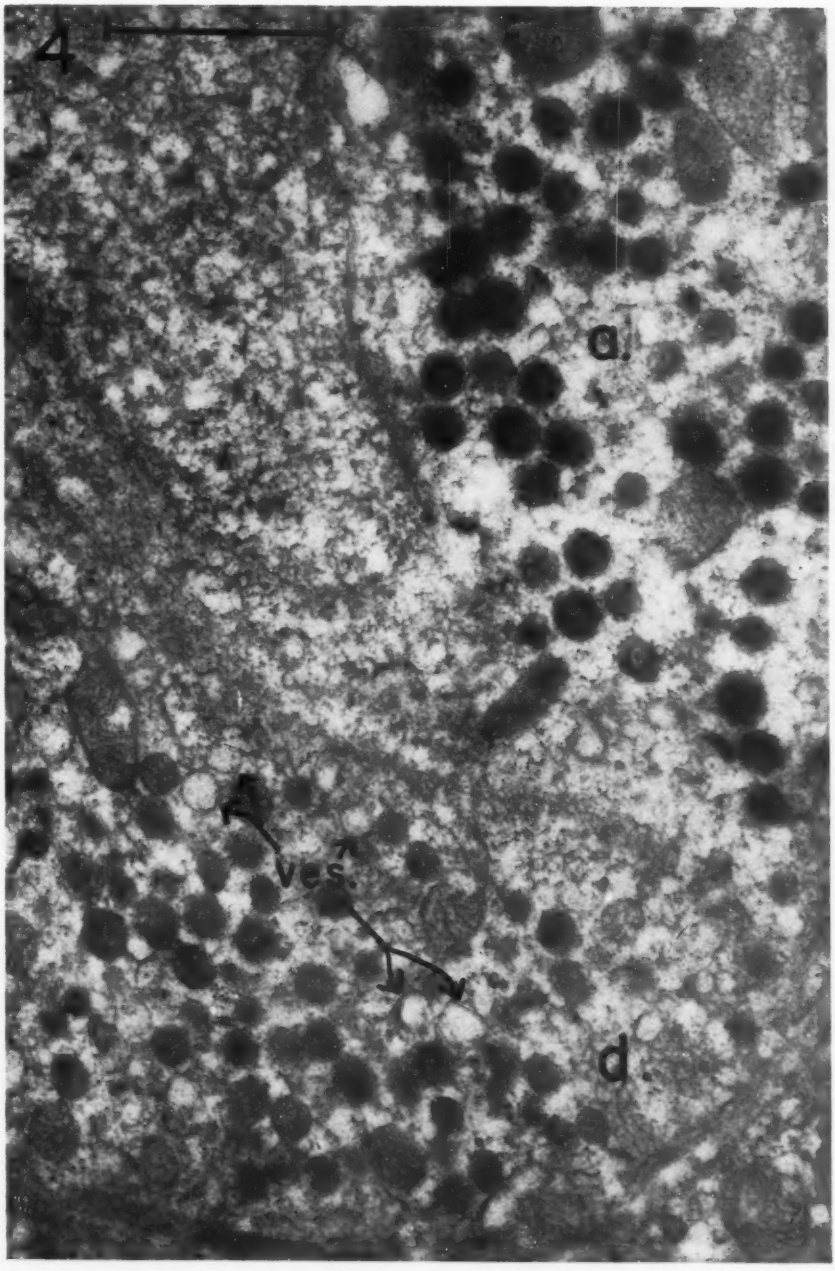
A wide variation in the granular content of beta cells is observed in the guinea pig. Some cells are seen packed with granules (Fig. 7) while others contain very few (Fig. 6). These differences exist between cells of a single islet as well as from one animal to another. It is tempting to think that these variations may depend upon differences in the secretory cycle, although comparable variations are not observed in the islets of the cat. In all of our cats, the beta cells show a high concentration of granules (Fig. 1).

Beta cells of the guinea pig have a marked polarization. The greatest concentration of granules is at the vascular pole, whereas the well-developed Golgi zone (Fig. 6) and the greatest concentration of endoplasmic reticulum tend to be at the opposite pole. In these respects, the cell differs markedly from the alpha cell. In the cat, the polarity of these systems is not as well developed as in the guinea pig.

Many tiny granules pervade the beta cell cytoplasm, giving it a stippled appearance (Fig. 7). The size and density of these granules make it likely that they are RNA granules (10). We have the decided impression that these granules are more numerous in beta cells than in alpha cells.

Mitochondria are the only organelles that appear similar in all respects in both alpha and beta cells. With this exception, quantitative or qualitative differences in the organelles of these two cell types are to be emphasized, and intergrades between the cells are never seen.

FIG. 4. Cat. In the upper part of this figure is the cytoplasm of an alpha cell (*a*). In the lower part is that of a "granulated delta cell" (*d*) as described in the text. The contents of the latter's granules themselves are finely granular and are of relatively low density. Its cytoplasm is vesiculated (ves.) like that of the typical delta cell.


4
a
Ves.
d

Delta Cells. Delta cells were particularly sought in the cat because both alpha and beta cells are always richly granulated and both possess distinctive characteristics. A fairly rare cell with nongranular cytoplasm is to be found, and presumably represents the delta cell (Figs. 1, 5). A fourth type of cell is also present occasionally, having a similar cytoplasm but containing specific granules distinguished from those of alpha cells by their low density and internal structure (Fig. 4), and from granules of the beta cells by their spherical form. These are probably to be regarded as "granulated delta cells" (11).

The cytoplasm of the delta cell is distinctive when compared with that of alpha and beta cells (Fig. 1). If one ignores the specific granules, the overall protoplasmic density is greater than that of either of the other two cell types. The cytoplasm appears to contain numerous "vacuoles" or "vesicles" containing an amorphous material of appreciable electron density (Fig. 5). The encircled zone of Figure 5 indicates where this pattern can be seen to advantage. The "granulated delta cells" (Fig. 4) have an identical cytoplasm in which vesicles are to be seen together with the granules. Vesicles and granules are of the same general size and it seems likely that the latter may be derived from the former. The substance of the granules is itself apparently finely granulated or vacuolated, and in this respect this granule differs from that of alpha cells.

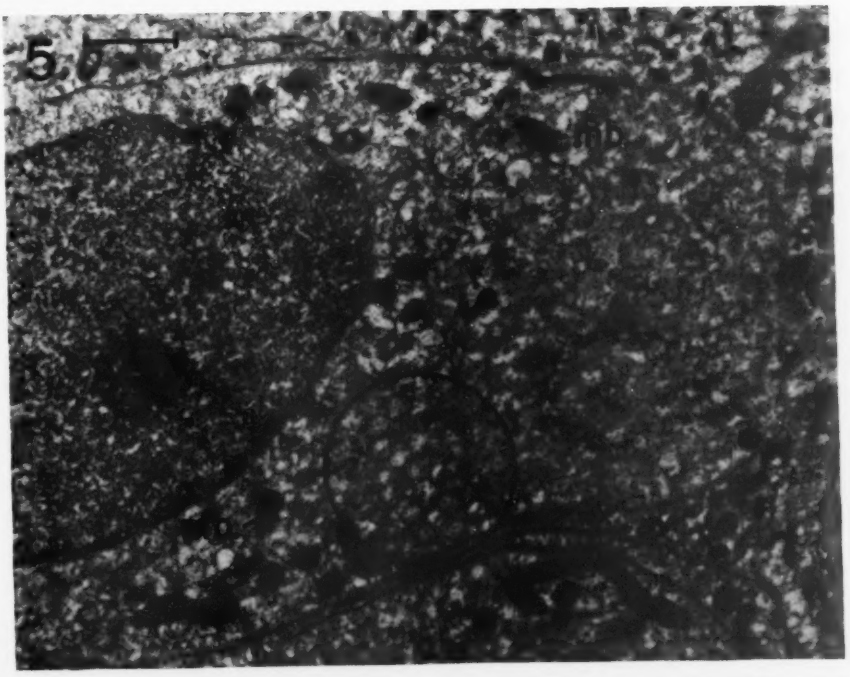
The delta cells of the cat also contain occasional elongated dense bodies similar in size to mitochondria (Figs. 1, 5, *mb.*). These lack internal structure and may be the equivalent of "microbodies" as described in other cell types (12).

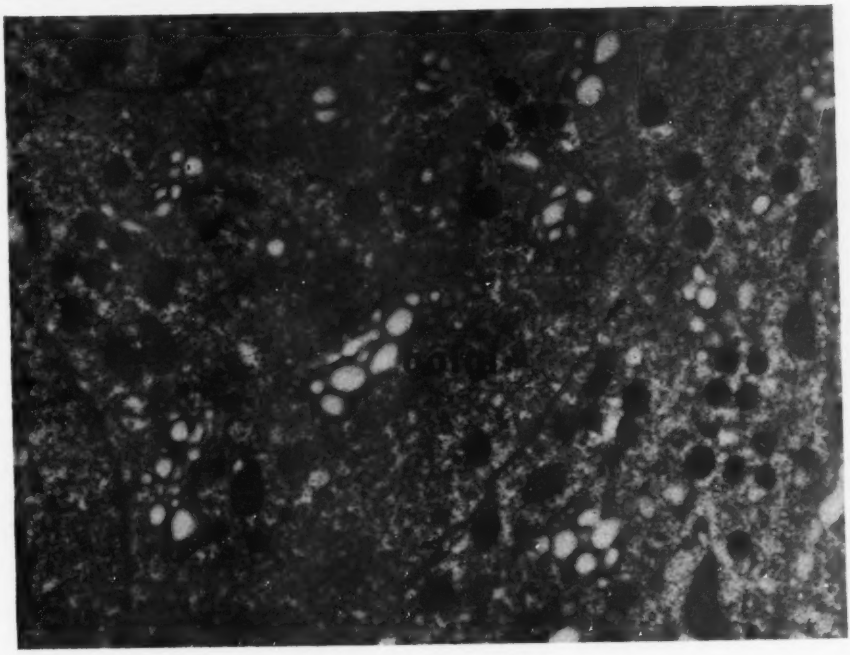
The Golgi apparatus and endoplasmic reticulum are poorly developed in the delta cell. There is but a sparse distribution of RNA granules. The scattered mitochondria are not distinguishable from those of the other cell types.

Occasional nongranulated cells are to be seen in the islets of guinea pigs. In this species, though, there is the possibility of confusing delta cells with poorly granulated beta cells or, perhaps, even with the gamma cells that have been described in this species, and which probably represent intra-islet duct cells (11). For these reasons we confine our description of the delta cell to the cat. Our findings are in general agreement with Lacy (2) and with Lacy's interpretation as published by Hartroft (3).

FIG. 5. Cat. Delta cell showing the characteristically vesiculated cytoplasm (particularly circled area) but no specific granules. Characteristic dense bodies (mb.) the size of mitochondria are found typically in these cells. (These may represent "microbodies" as described by others.)

FIG. 6. Guinea pig. Beta cell cytoplasm containing in this instance rather few specific granules (compare with Figure 7). Numerous Golgi vacuoles are apparent.


5 μ



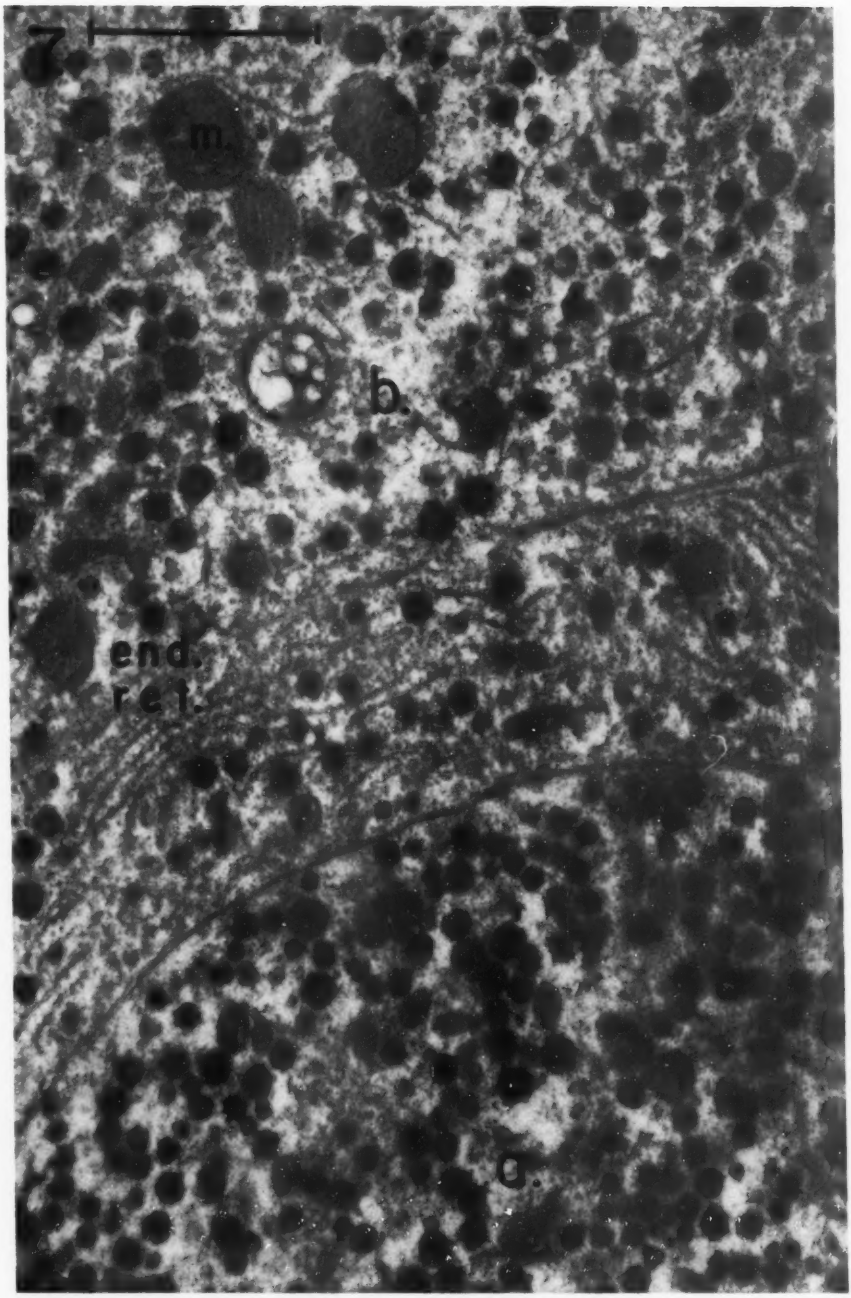
Vascular Relations of Islet Cells. Capillaries associated with small amounts of connective tissue penetrate through the pancreatic islets. The capillary wall, discussed in detail below, consists of an endothelial sheet resting upon a thin basement membrane (Fig. 9, *b.m.*). As is apparent in Figure 8, there is also a thin basement membrane (*b.m.*) between the stroma and the parenchymal cells. At times (but by no means always) the vascular pole of beta cells is specialized in being more or less elaborately folded, thus producing "pseudopods" (*p. pod*). Between the pseudopods, and within the parenchymal basement membrane, "subcellular spaces" (*subcell. s.*) may be conspicuous. The folding of the basal cell surface may be regarded as providing a mechanism increasing the area available for metabolic exchange, and resembles basal anatomical features described in the kidney (13, 14), placenta (15) and in some other endocrine glands (16). Since the "subcellular space" often is not patent, it may have only a transitory existence in life, or it might even be only a potential space expanded as an artifact during fixation.

Typical nonmyelinated nerve fibers are commonly seen in the connective tissue spaces in close association with blood vessels. Schwann cells accompany them. It has not been possible to determine with certainty whether or not such autonomic nerve fibers come into intimate contact with, or terminate upon, the parenchymal cells. Structures resembling naked nerve fibers are seen occasionally within the parenchymal basement membrane and in intimate contact with secretory cells. It is difficult, however, to preclude the possibility that these may be isolated pseudopods of neighboring beta cells rather than being nerve processes. They lack "synaptic vesicles" or "synaptic membranes" which might otherwise positively identify them.

Neuro-insular complexes consisting of an intimate association of islet and sympathetic ganglionic cells have been described (17) as exceptional structures in the midst of exocrine acinar tissue. On a single occasion we observed one of these. What were clearly islet cells were embedded in an autonomic ganglionic mass. Unfortunately, the exact relation between nerve processes and islet cells could not be determined for the plane of section was unfavorable for delimiting exactly most of the cell boundaries.

The Capillary Wall. The capillaries of the pancreatic islands consist in most places of an attenuated endothelial sheet. At high magnifications this often shows interruptions (Fig. 9). When viewed in the horizontal plane tangential to the capillary surface (Fig. 10), the endothelium is seen to be penetrated by a system of pores approximately 0.05 μ in diameter. Comparable endothelial pores of the peritubular capillaries of the kidney studied by Pease (18) were 0.06 μ in diameter. Similar fenestrations of the glo-

FIG. 7. Guinea pig. The cytoplasm of a beta cell (*b*) and adjacent alpha cell (*a*) is compared. The greater density of the granules of the alpha cell is apparent. The beta cell cytoplasm has an extensive development of endoplasmic reticulum partly visible here and with, perhaps, more tiny RNA granules than the alpha cell.


m.
b.
end.
ret.
c.

merular capillaries were approximately twice as wide. The question as to whether or not these fenestrations exist in life or are artifactual was considered in some detail, and it was concluded that they probably have a true existence. Their existence may, of course, be transitory.

The capillary basement membrane consists of a dense lamina (Fig. 9), and a layer of low density between this and the endothelial sheet. The latter zone has been called a "cement layer" (18). It is reasonable to think that the dense lamina provides the structural support for the capillary. The fenestrations of the endothelial sheet are of such a size that they would not constitute a barrier to diffusion processes. Thus, the only continuous barrier between plasma and connective tissue fluid would seem to be the basement membrane.

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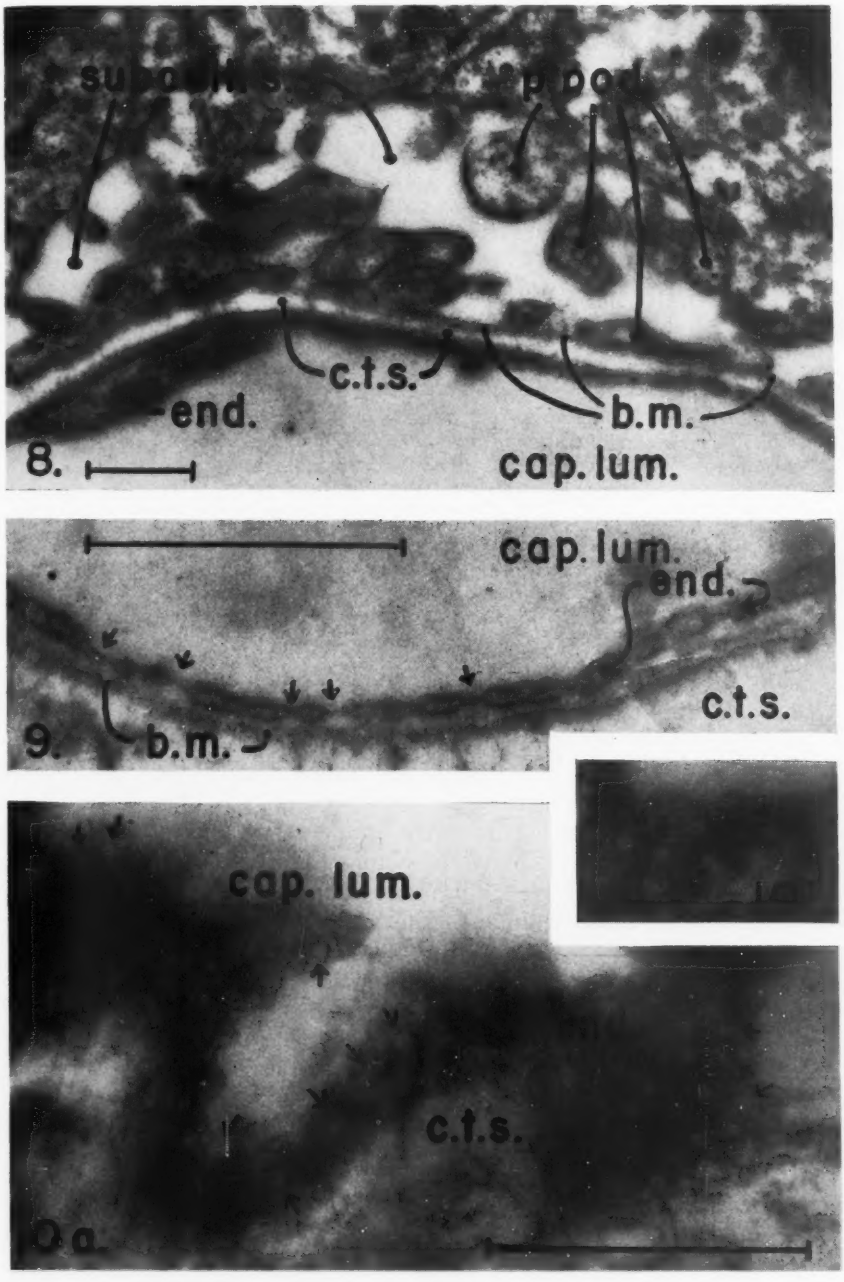
FIG. 8. Guinea pig. A view showing relations between parenchymal cells and the vascular bed. The capillary lumen (*cap. lum.*) is indicated. The endothelial portion (*end.*) of the capillary wall is visible. There is a connective tissue space (*c.t.s.*) followed by the parenchymal basement membrane (*b.m.*). In this area, the basal portions of beta cells are folded to produce pseudopods (*p. pod*) which extend into a subcellular space (*subcell. s.*).

FIG. 9. Guinea pig. High magnification of a transverse section through a capillary wall. The endothelium (*end.*) is indicated. Arrows point out some of the interruptions in this sheet. The capillary basement membrane (*b.m.*) is visible. A low-density "cementing layer" between the capillary basement membrane and the overlying endothelium can be seen.

FIG. 10a, b. Guinea pig. Nearly horizontal views of capillary surfaces at high magnification. The dense material is the endothelial layer of the capillary wall. Arrows indicate where pores through this sheet may be seen to advantge. Note the regularity of size and spacing.


c.t.s.
end.
b.m.
cap. lum.
8.
cap. lum.
end.
c.t.s.
9.
b.m.
cap. lum.
c.t.s.
9a.

INFLUENCE OF INSULIN AND CORTISONE ON HEPATIC AND ADIPOSE TISSUE METABOLISM OF RATS[1]

FRANZ X. HAUSBERGER

Daniel Baugh Institute of Anatomy, Jefferson Medical College of Philadelphia, Philadelphia, Pennsylvania

ABSTRACT

Rats received 2.5 mg. and 5 mg. of cortisone acetate and/or various amounts of protamine zinc insulin for 4 and 14 days, respectively. Insulin produced markedly accelerated oxidation of, and lipogenesis from, radio glucose by surviving liver and adipose tissue. The effect of liver was temporary; adipose tissue metabolism remained elevated throughout the experiment. Pre-treatment with cortisone diminished oxidation and lipogenesis in both tissues. After simultaneous administration of both hormones, the insulin effect was the prevailing one. Insulin stimulated adipose tissue metabolism *in vitro* to the same degree as after administration *in vivo*. It is theorized that the adiposity seen in hyperadrenocorticism may be caused by the prevailing effect of insulin on adipose tissue growth and adipose tissue lipogenesis.

IN AN earlier communication it was shown that insulin administration accelerates fat deposition in, and growth of, adipose tissue. This effect is only moderately and inconsistently diminished by simultaneous cortisone injections (1). The increased food consumption observed in such animals could have caused augmented fat storage. On the other hand, direct stimulation of fat cells by insulin seems conceivable, since pre-treatment with this hormone greatly enhances lipogenesis by liver (2) and adipose tissue (3). Adrenocorticosteroids are known to suppress lipogenesis in rats (4, 5). Histological evidence indicates compensatory increased release of either hormone in the presence of excess amounts of the other, antagonistic, one. Therefore, comparative studies were performed in order to determine the effect of combined insulin and cortisone administration on hepatic and adipose tissue metabolism. Lipogenesis from, and oxidation of, radioglucose were investigated in rats treated for different periods of time with either hormone, or both hormones together. In addition, the effect of insulin added to surviving liver and adipose tissue were studied.

EXPERIMENTAL

Male Wistar rats, weighing between 240 and 300 gm. at the onset of the experiment and showing a daily weight gain of at least 2.5 gm., were used. They were fed Purina Laboratory Chow and were weighed daily.

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In the first part of this investigation, Cortisone acetate, 2.5 mg. and 5 mg., respectively, and Protamine zinc insulin (Lilly) were administered once per day for different time periods. The last injection was given about two hours before sacrifice. The rats were sacrificed by skull fracture, exsanguinated, and the tissues processed rapidly. Pooled hepatic and adipose tissues of 3 rats were used in each experiment; these were incubated in Krebs-Henseleit buffer containing 400 mg.% of glucose for 3 hours at 37 degrees Centigrade. The same incubation and analytical methods were used as in an earlier investigation (3). Most experimental animals received 5 mg. of cortisone and/or 12 units of insulin for periods of 4 or 14 days, respectively. Only the results obtained from this group are shown in Table 1.

In the second part of this study, liver and adipose tissue from fed rats were incubated in the same manner, and varying amounts of crystalline insulin were added to the medium. The experimental and analytical procedures were the same as in the first part.

RESULTS

A. *Incorporation of glucose carbon into CO_2 and fatty acids*

Insulin: Insulin was administered in a dosage of from 4 to 80 units per day. Increased gain of body weight was noted in all animals, the gain being usually more marked and more consistent in rats receiving more than 12 units of insulin. This higher dosage, however, was well tolerated only during the first 2 or 3 days. Thereafter, many animals receiving only insulin succumbed to hypoglycemic attacks although 5% sucrose was added to the drinking water. Two groups of 3 rats each received 4 units of insulin for 4 and 14 days, respectively. Twelve other rats were injected with 40 and 80 units; only two of these animals survived. In addition, 3 animals received 12 units for 42 days. Lipogenic activity in rats receiving the higher or lower dosage did not differ significantly from that observed in animals injected with 12 units for 4 and 14 days, respectively. These latter values are shown in Table 1.

Hepatic oxidation and lipogenesis were considerably accelerated after pre-treatment with insulin for 4 days. Continuation of hormone administration, however, always resulted in a return to normal levels of activity.

Adipose tissue normally utilizes less glucose for oxidation than liver, but more than ten times as much for lipogenesis. Pretreatment with insulin produced a marked increase of both lipogenesis and oxidation, which was of approximately the same order of magnitude throughout the entire experimental period. The values after 42 days were within the same elevated range.

Cortisone: In addition to the animals injected with 5 mg. of cortisone, 4 groups of 3 rats each were injected with 2.5 mg. of cortisone daily for 4 and 14 days, respectively. Gain of body weight was markedly reduced by the higher cortisone dose, and slightly less so by the administration of 2.5 mg.

Administration of 5 mg. of cortisone daily for 4 days diminished hepatic lipogenesis to approximately a third of the norm, oxidation slightly less. Prolonged steroid administration did not change the diminished rate of

TABLE 1. MICROGRAMS OF GLUCOSE CARBON INCORPORATED INTO CARBON DIOXIDE (CO_2) AND FATTY ACIDS (FA) BY ONE GRAM OF LIVER (L) AND ADIPOSE TISSUE (AT) OF RATS

Utilization of 104 micrograms of glucose carbon is equivalent to one per cent. The experimental animals received 12 units of protamine zinc insulin and/or 5 mg. of cortisone for 4 and 14 days, respectively. Each experiment was performed with pooled tissues of 3 rats. The heading for the column "wt. gain" refers to the average daily weight gain expressed in per cent body weight at the beginning of the experiment.

Controls					Insulin					Cortisone					Cort.-insulin				
Weight gain %	CO_2		F.A.		Weight gain %	CO_2		F.A.		Weight gain %	CO_2		F.A.		Weight gain %	CO_2		F.A.	
	L.	AT.	L.	AT.		L.	AT.	L.	AT.		L.	AT.	L.	AT.		L.	AT.	L.	AT.
					4 days														
0.9	375	240	3.0	170	3.3	1830	576	107	518	−0.7	47	95	3.6	7.3	0.8	1050	637	78	308
1.1	347	185	3.7	128	2.8	1060	634	96	307	−0.3	101	64	1.7	12.0	1.5	1540	634	82	339
1.0	485	220	2.9	60	2.8	923	297	94	566	−1.2	588	222	0.3	13.0	1.4	889	259	16	206
1.2	767	140	3.7	66	2.5	656	594	68	651	−1.1	519	153	1.8	21.0	1.1	889	265	55	157
1.0	387	180	11.0	85	3.0	164	986	7.6	1195	0.42	197	64	0.9	5.2	2.0	324	107	2.0	80
1.1	893	139	15.0	58						0.42	217	63	0.3	8.0	2.0	163	585	2.4	339
1.0	445	92	11.0	12						0.38	315	131	7.0	54.0	2.8	192	520	1.0	113
1.0	490	274	1.3	7.6						0.82	318	96	3.8	20.0					
	524	184	6.5	82		927	617	75	647		288	111	2.4	17.5		721	430	34.0	220
					14 days														
					1.3	274	375	4.4	417	−0.8	283	127	3.9	12.0	1.3	493	469	4.0	514
					3.0	1099	841	5.1	581	0.0	357	101	7.0	11.0	1.3	304	255	4.0	212
					2.6	375	594	5.0	473										
						538	603	4.8	490		320	114	5.5	11.5		399	362	4.0	363

oxidation, and lipogenesis showed a not significant increase. Adipose tissue lipogenesis was reduced to one-fifth of the norm after 4 days, oxidation not quite as much. A further but not significant decrease was noted after 14 days (Table 1).

Daily administration of 2.5 mg. of cortisone did not influence hepatic lipogenesis and oxidation within the 4 day period. Adipose tissue oxidation, however, was reduced to 30%, and lipogenesis to 60% of the norm. Pretreatment for 14 days lowered glucose utilization for both metabolic processes in either tissue to the same level as did administration of 5 mg. of cortisone.

Cortisone and insulin: The results of the experiments in which 5 mg. of cortisone together with 12 units of insulin were administered daily are shown in Table 1. In some additional groups of 3 rats each, variable amounts of insulin were administered, the dosage of cortisone remaining the same. Two groups received 4 units of insulin each, and two other groups up to 120 units of insulin for 4 days. This latter amount was administered in gradually increasing dose, 120 units being injected on each of the last 2 days of the experiment.

Rats receiving 4 units of insulin showed only slightly accelerated weight gain; 120 units produced a considerably accelerated weight gain, which amounted to approximately 16% of the weight at the beginning of the experiment.

Despite the different weight gain and insulin dosage, essentially the same results were observed in all groups. Hepatic lipogenesis and oxidation showed marked variations; the average values were above the norm after 4 days of treatment, and remained within the normal range after 14 days. Adipose tissue demonstrated markedly enhanced oxidative and lipogenic rates. Although the values observed were partly lower than in animals receiving only insulin, they were still 3 to 4 times higher than in controls. No significant decrease was observed after prolonged treatment. Similarly as in the liver, no relationship between weight gain and tissue activity was observed.

B. *Effect of insulin on tissue metabolism in vitro*

Eighteen experiments were performed in which insulin was added to the medium. Concentrations of 0.3 to 2.5 units of crystalline insulin per milliliter of medium were used. The glucose concentration in these experiments was 100 mg% which explains the lower metabolic values. Duplicate determinations of oxidation and lipogenesis by normal tissues rarely showed a variation of more than 10 to 15%. Occasionally, differences up to 50% were observed, possibly due to damage to the tissues during preparation. Therefore, no changes below 50% were considered significant. Figure 1 shows the result of two typical positive experiments. A positive response of adipose tissue, which was observed in 11 experiments, was always pro-

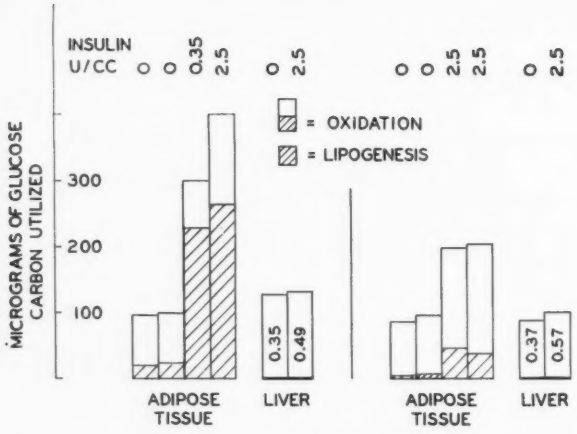

FIG. 1. Glucose utilization by liver and adipose tissue *in vitro*, without and with addition of insulin. Pooled tissues from 3 rats, i.e. 200 mg. from each, were used in each experiment. The values for hepatic lipogenesis are expressed by numbers. The lower lipogenic activity, as compared to Table 1, is due to the lower glucose concentration employed (100 mg. %).

nounced and within the same order of magnitude as after pretreatment with insulin. Furthermore, lipogenesis was always more affected than oxidation. The response by liver was, according to our conditions, not significant although a slight increase in oxidation and lipogenesis was sometimes observed. This part of our study is being continued.

DISCUSSION

Hepatic lipogenesis from glucose in normal rats was low. Chernick and Chaikoff (2) found 30 times higher values. This difference is due to the fat-free diet employed by these investigators, as pointed out earlier (6). Adipose tissue lipogenesis was more than 10 times higher, as already observed in earlier investigations (2, 6). These findings compare well with those of Favarger and Gerlach (7). In their investigation, mice receiving glucose intravenously incorporated, within 12 minutes, about 14 times more glucose carbon into fatty acids of adipose tissue than into liver fatty acids.

Insulin enhanced hepatic lipogenesis only temporarily and inconsistently while adipose tissue lipogenesis remained accelerated during the entire period of insulin administration. Osborn *et al.* (8) also observed a temporary effect of insulin on liver lipogenesis, with a maximum of activity on the fifth day.

The results of this study confirm the inhibitory effect of cortisone on lipogenesis observed by earlier investigators. Simultaneous insulin administration not only overcame this inhibition but the insulin effect remained the prevailing one. These findings are in agreement with a report

by McNaught *et al.* (9) on the *in vivo* effect of cortisone and hydrocortisone, and insulin, on lipogenesis from glucose by mammary gland tissue. Similarly, Kupperman *et al.* (10) failed to observe inhibition of the hypoglycemic effect of insulin by hydrocortisone.

Addition of insulin to adipose tissue produced inconsistent effects during the three hours of the experimental period. However, insulin administration to rats fasted overnight accelerates adipose tissue lipogenesis markedly (11), an observation which excludes increased food intake as a cause of the changes in lipogenic activity. There are several other findings which also suggest a direct effect of insulin on fat cells. Sano (12) reported increased glycogen formation after addition of insulin to surviving adipose tissue, and Krahl (13) noted accelerated glucose uptake under similar conditions. Increased fat deposition at the site of insulin injections was seen by Renold *et al.* (14) and Sidman (15) found enhanced glycogen and fat formation after adding insulin to adipose tissue cultures.

It was shown in an earlier study that insulin administration, even in the presence of excess cortisone, promotes increased growth of, and accelerated fat deposition in, adipose tissue (1). The results of this investigation indicate that the latter process is at least partly caused by accelerated new formation of fat *in loco*.

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THE EFFECT OF CHANGE IN CHEMICAL STRUCTURE OF SOME THYROXINE ANALOGUES ON THE METAMORPHOSIS OF *RANA PIPIENS* TADPOLES[1]

WILLIAM L. MONEY, ROBERT I. MELTZER, JOYCE YOUNG AND RULON W. RAWSON

Sloan-Kettering Institute for Cancer Research of the Memorial Center, New York, New York, and the Research Division of Warner-Chilcott Laboratories, Morris Plains, New Jersey

ABSTRACT

Forty-three different substances structurally related to thyroxine were assayed for their metamorphosing effect on *Rana pipiens* tadpoles. Triiodinated compounds with iodines at the 3,3′,5 positions were found more active than tetraiodinated compounds. The 3,5- and 3,3′ diiodinated compounds tested also had some activity except for the 3,5 diiodothyroformic acid. None of the monoiodinated compounds assayed showed biological activity. The most active compound studied was 3,3′,5-triiodothyropropionic acid which was 300 times more potent that *l*-thyroxine.

IN 1912 Gudernatsch (1) showed that tadpole metamorphosis was under the control of the thyroid gland. Since these original observations, many iodinated compounds have been reported capable of inducing amphibian metamorphosis. Recently the relationship of thyroxine-like metamorphosing activity and chemical structure of some compounds has been studied by Roche *et al.* (2) and reviewed by Bruice *et al.* (3).

The recent discovery of naturally occurring thyroxine-like compounds has stimulated research on analogues and their effect on tadpole metamorphosis. It seemed, therefore, of some importance to undertake a study to relate chemical structure of thyroxine analogues to their biological activity as manifested by amphibian metamorphosis. The results of a study using some thyroxine analogues and *Rana pipiens* tadpoles are presented in this report.

MATERIALS AND METHODS

Tadpoles were obtained by experimentally induced ovulation and fertilization (4, 5). The animals were reared in spring water at room temperature and fed a diet of boiled spinach. Overcrowding was avoided as much as possible and water changed whenever it became cloudy. When the tadpoles reached the desired length they were used for assay of the test compounds. All assays were done in a constant temperature (72–74° F). The assay was essentially that of Shellabarger and Godwin (6), only slightly modified

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for our purposes. Usually 10 tadpoles were used for each dilution of the test solution. The test compounds were assayed at levels of between three and seven different concentrations. In all, approximately 6,000 tadpoles were used for assay of the different compounds.

The compounds used consisted of 43 different substances synthesized mainly in the Warner-Chilcott Laboratories.[2] These compounds were all diphenyl ethers structurally related to thyroxine.

EXPERIMENTAL CONSIDERATIONS AND RESULTS

The validity of results obtained in the bio-assay of compounds used in minute quantities and dilute concentrations is limited by several factors. From the literature it is apparent that these limiting factors have not always been considered in assays using tadpoles. It would seem worthwhile, therefore, to point out some of the problems which make evaluation of such data difficult. The first consideration is the solubility of the compound being assayed. The preparations used in this report were frequently soluble in NaOH, HCl or ethyl alcohol, but some were not soluble in any of these. When the latter was the case it was necessary to add the material as a suspension to the water in which the tadpoles lived. However, even soluble compounds may not have remained in solution after being added to the beakers containing the tadpoles. Indeed this appeared to be true with some samples used in the present studies. It would follow, then, that if compounds precipitated out of solution, they might have had less chance of exerting a biological effect on the tadpoles and the results of such assays, particularly the quantitative value, would be open to question. The second important consideration concerns the problem of the stability of the compounds either in stock solutions or after being added to the tadpole bath. We have found that the biological activity of some compounds changed rather markedly in stock solution even when maintained in the dark and cold. If such were the case, it naturally follows, that assay of unstable compounds would lead to spurious results and unjustified conclusions. The third and perhaps most serious error in evaluating comparative assays of the present type stems from the degree of purity of the compounds being tested. The contaminant present could produce a fantastic error in calculating biological activity. On the other hand, contaminants with low biological activity would result in values much less than actually present. In this report we are reasonably certain contamination existed in some compounds. Most of the compounds produced by the Warner-Chilcott Laboratories appeared on the basis of paper partition chromatography to have relatively little contamination. The 3,3′,5-triiodothyroacetic acid, 3,3′,5-triiodothyroformic acid and the 3,3′,5-triiodothyropropionic acid may have contained as much as 5–10% impurity. With these limitations in mind the present study is being reported. Whenever there was reason to question either the solubility, stability, contamination or the results of the assays, this has been indicated below. The calculated values, shown in the figures, indicate the biological effectiveness of the compounds relative to *l*-thyroxine which was given an arbitrary effectiveness equal to 100. This calculated potency is based on either the actual amount of the compound used, or a calculated amount, by weight, from a series of dilutions assayed at the same time. This amount of compound would result in a 20–25% average decrease in tadpole body length after 10 days of treatment, and would be equivalent to 5–6 micrograms of *l*-thyroxine.

[2] Smith, Kline and French Laboratories furnished the 3,3′,5-triiodo-*l*-thyronine. Travenol Laboratories, Inc. supplied the *l*-thyroxine. The 3,5-diiodo-thyrobutyric acid, 3,3′,5-triiodothyrobutyric acid and 3,3′,5,5′-tetraiodothyrobutyric acid were obtained from Cyclo Chemical Company. The 3,3′,5,5′-tetrachloro-*d*,*l*-thyronine was furnished by Doctor J. Lerman and synthesized by Sir C. R. Harington.

Compounds have been considered inactive if they produced no biological response when tested at a maximal dose of 500 μg. Such a dose would be 90–100 times the dose of *l*-thyroxine required to produce the desired 20–25% reduction in tadpole length. In the case of compounds listed as inactive, the growth of tadpoles was similar to that of untreated control animals.

For the sake of simplicity the data are presented on the basis of their relationship to the basic molecular configuration. The arrangement of the compounds in the figures is purely arbitrary and does not imply conversion of one compound into another. Most of the compounds listed do not exist in optically active forms. For those which do have optical isomers indication has been made below as to which forms were used.

I. RELATIVE METAMORPHOSING ACTIVITY OF THE THYRONINE SERIES

Figure 1 shows that four of the nine thyronine compounds assayed were completely inactive. The only compound more active was 3,3′,5-triiodo-*l*-thyronine, which was five times more active than thyroxine. The reverse

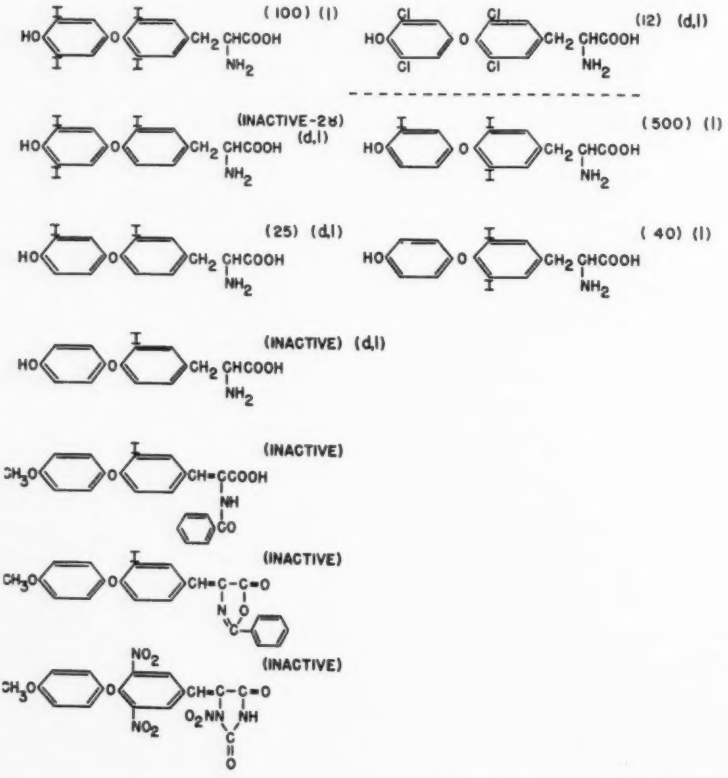

FIG. 1. The relative metamorphosing activity of the thyronine series. The first numbers in brackets indicate the calculated biological potency. The second bracket indicates the isomer used (either l,d, or d,l).

triiodo-*d,l*-thyronine (3,3′,5′) was inactive at the dose tested (lack of animals prevented testing at higher doses). This compound, however, has been reported to show 1/20 the activity of *d,l*-thyroxine (2). The difference in activity between 3,3′-diiodo-*d,l*-thyronine and 3,5-diiodo-*l*-thyronine may be due to the fact that the former was in the racemic form while the latter was present as the *l*-form. If one assumes that the *d*-isomer is inactive then the results of both assays would be essentially identical. Whether or not this assumption is justified is still an open question from our data. On the basis of unpublished studies we are inclined to feel that under certain conditions the *d*-form may be active. 3,3′,5,5′-tetrachloro-*d,l*-

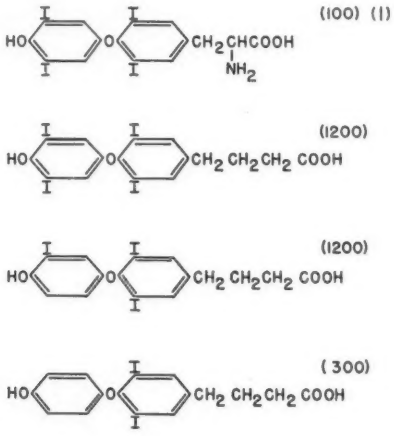

FIG. 2. Relative metamorphosing activity of the thyrobutyric acid series.

thyronine had some activity, but the results are considered questionable because of uncertain stability of the compound in solution.

II. RELATIVE METAMORPHOSING ACTIVITY OF THE THYROBUTYRIC ACID SERIES

At present only three compounds of this series have been assayed (Fig. 2). In all three, the results must be considered questionable owing to the limitations discussed above, particularly since paper partition chromatography showed these samples to be contaminated. (The exact nature of these contaminants has not been determined.) On the basis of these data (in Fig. 2), however, it would appear that both the tetra- and triiodinated forms have the same amount of activity. The diiodinated compound appears more active than thyroxine but less so than the other two compounds. It is obvious that additional determinations of related compounds must be made to evaluate the effectiveness of these substances.

III. RELATIVE METAMORPHOSING ACTIVITY OF THE THYROPROPIONIC ACID SERIES

As may be seen in Figure 3, fourteen structurally related compounds of this series were compared to thyroxine. Half of these were considered inactive while the others showed various degrees of potency. The biological activity ranged from essentially trace amounts to the most active compound, 3,3′,5-triiodothyropropionic acid which was 300 times more potent than thyroxine. Moreover, the tetraiodinated form is also highly active, and both the 3,5- and 3,3′-diiodothyropropionic acid compounds show greater activity than thyroxine. In contrast to the highly active 3,3′,5 compound the reverse form (3,3′,5′) appears to be low in potency. The ethyl β-[3-amino-4-(4′-methoxyphenoxy)-phenyl]-propionate appears more

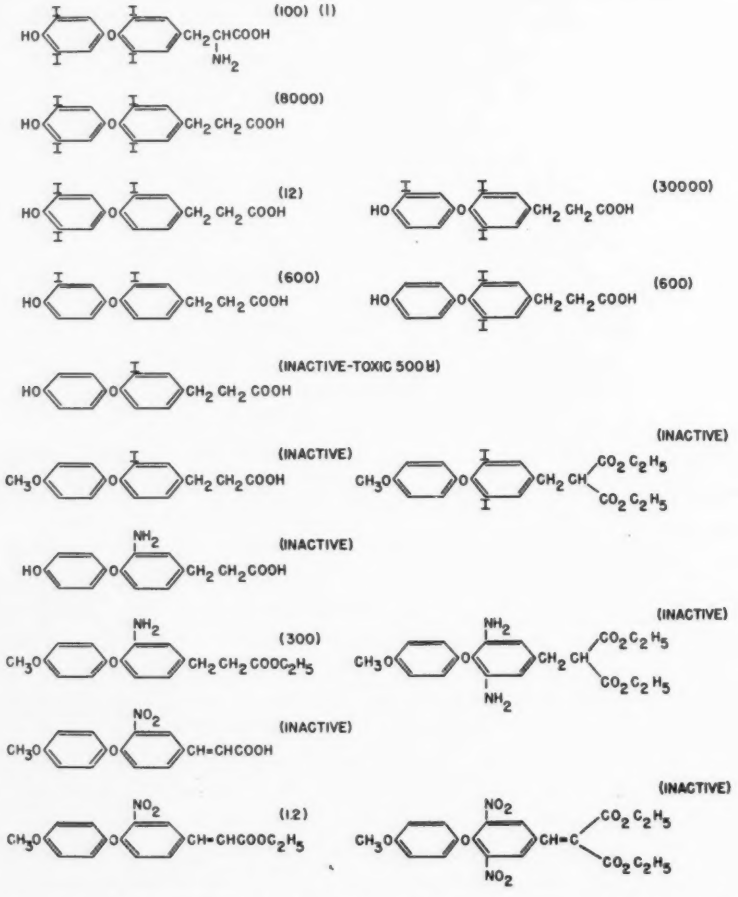

FIG. 3. Relative metamorphosing activity of the thyropropionic acid series.

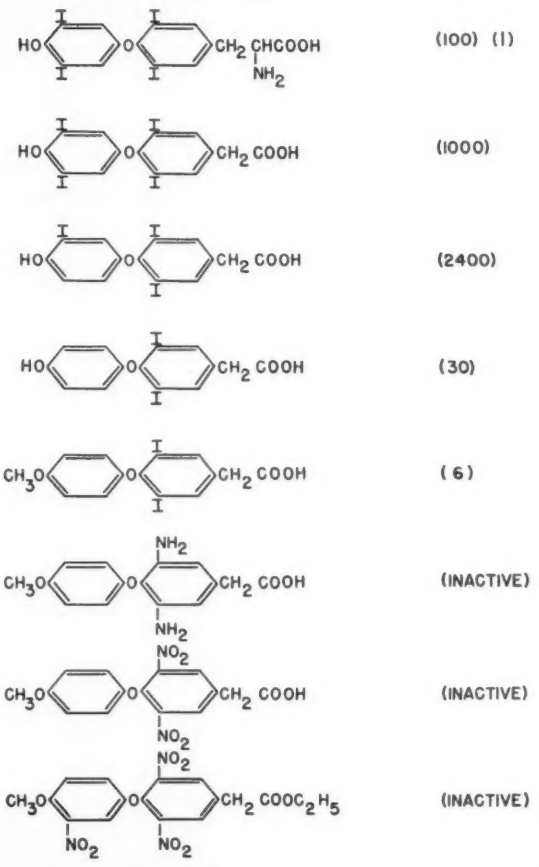

FIG. 4. Relative metamorphosing activity of the thyroacetic acid series.

active than thyroxine, but the validity of these results is uncertain because of solubility difficulties with the former compound. It might be noteworthy that this compound contains no halogen. A small amount of biological activity was present in the ethyl 3-nitro-4-(4′-methoxy-phenoxy)-cinnamate when tested at relatively high doses.

IV. RELATIVE METAMORPHOSING ACTIVITY OF THE THYROACETIC ACID SERIES

Seven different compounds of this series were compared with thyroxine for biological activity (Fig. 4). Three of these were inactive when tested at the maximal dose of 500 μg. The other compounds showed variable biological potency. The triiodinated material was 24 times and the tetraiodinated 10 times more active than thyroxine. The 3,5-diiodothyroacetic acid preparation proved to be about 1/3 as active as thyroxine. Substitu-

tion of a methoxyl group to replace the hydroxyl at the 4′ position of this compound appeared to decrease the biological activity even further.

V. RELATIVE METAMORPHOSING ACTIVITY OF THE THYROFORMIC ACID SERIES

Of the nine compounds tested in this series (Fig. 5) only the tetra- and triiodinated thyroformic acids showed significant biological activity. The

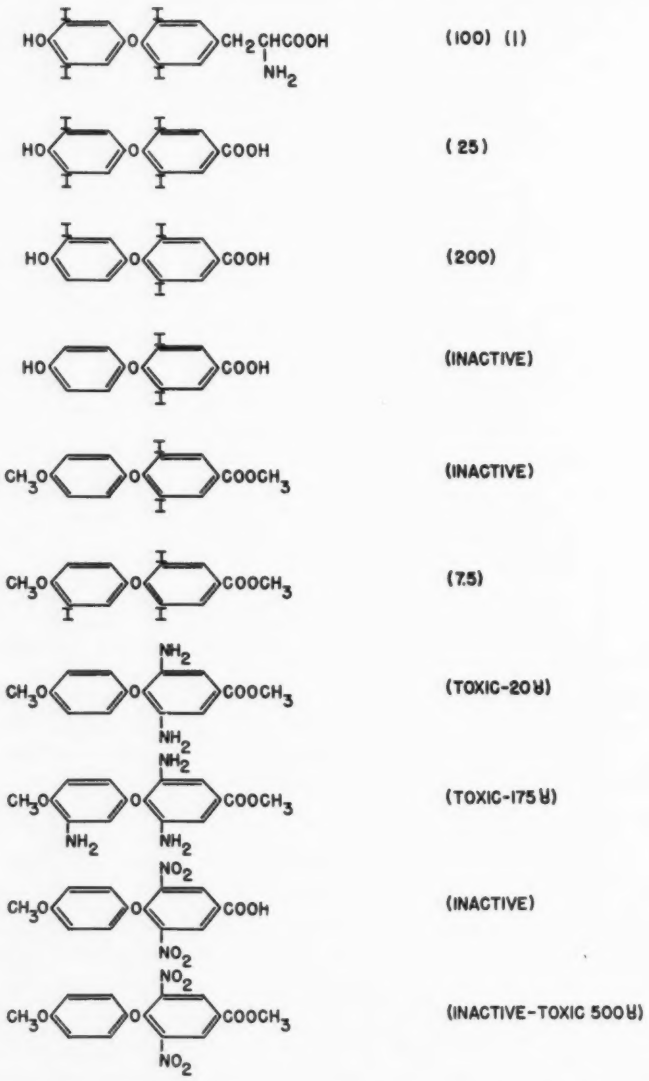

FIG. 5. Relative metamorphosing activity of the thyroformic acid series.

latter was the only one which appeared more active than thyroxine. Methyl 3,5-diiodo-4-(3′-iodo-4′-methoxyphenoxy)-benzoate had less than 1/10 the activity of thyroxine. Three of the others were toxic and killed tadpoles in doses too low to determine biological activity.

COMMENTS AND CONCLUSIONS

The original objective of this study was an attempt to relate chemical structure of some thyroxine analogues to their biological activity as evidenced by amphibian metamorphosis. Certainly, many more analogues must be studied before an exact correlation can be made. However, certain conclusions apparently can be made on the basis of the data presented above. Triiodinated compounds appear more active than the tetraiodinated ones when the iodines are at the 3,3′,5 positions. It is not possible from the present data to state conclusively that the 3,3′,5′ triiodinated compounds are less active than thyroxine and the 3,3′,5 triiodinated compounds. Only two of the 3,3′,5′ compounds have been tested and both were less active than the 3,3′,5 compounds. Additional preparations must, however, be assayed before this point can be clarified.

In the present series of assays, when triiodinated compounds were active, the diiodinated compounds (either 3,5 or 3,3′) also had some activity, except for the 3,5 diiodoformic acid. Neither the monoiodothyronine nor the monoiodothyropropionic acid compounds showed activity. The activity of other monoiodinated compounds is not known and requires future assay.

In terms of activity per unit weight of material the most active compound tested was 3,3′,5-triiodothyropropionic acid which was 300 times more potent than *l*-thyroxine. The next most active compound was 3,3′,5,5′-tetraiodothyropropionic acid which was 80 times more active than thyroxine. The 3,3′,5-triiodothyroacetic acid and 3,3′,5,5′-tetraiodothyroacetic acid followed closely, being 24 and 10 times more active respectively than thyroxine.

In general our figures for potency agree quite well when comparisons can be made with data in the literature (2, 3, 7). Like others (2, 7), we find marked discrepancies between our values for some compounds and those previously reported (8). It is, however, quite possible that this difference is due to some of the limiting factors discussed above. Only additional observations will permit a complete answer to these discrepancies.

Acknowledgments

Miss Alice Glattstein and Miss Diane Feldman contributed valuable help in raising the tadpoles used in the study.

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STUDIES ON THE LOCI OF ACTION OF CORTICAL HORMONES IN INHIBITING THE RELEASE OF ADRENOCORTICOTROPHIN[1]

S. M. McCANN, ALLEN FRUIT AND BROOKE D. FULFORD

Department of Physiology of the University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

ABSTRACT

Compensatory adrenal hypertrophy is reduced in rats with diabetes insipidus as a result of hypothalamic lesions. Adrenal atrophy can be produced by injection of exogenous hydrocortisone into rats with diabetes insipidus. Rats with mild diabetes insipidus require less steroid to produce this effect than do normal rats, whereas rats with severe diabetes insipidus require more steroid than do normal rats. It is concluded that variations in cortical hormone concentration in the blood can still alter the release of ACTH in rats with diabetes insipidus though these rats may be less sensitive to such alterations than normal rats. Administration of a large dose of hydrocortisone acetate to rats diminished the antidiuretic response to a standard mild electric shock stimulus and decreased the antidiuretic hormone titer in jugular venous plasma from stressed rats. It was concluded that adrenal steroids block the release of ACTH from the pituitary by 1) a direct action on the pituitary gland, and 2) by decreasing the release of antidiuretic hormone, the presumed neuro-humor stimulating ACTH release.

THE classical studies of Ingle (1), Sayers and Sayers (2) and others (3, 4) have conclusively demonstrated that there is an inverse relationship between the concentration of cortical hormones in blood and the release of adrenocorticotrophin (ACTH). Thus, a markedly increased secretion of ACTH occurs in adrenalectomized animals, whereas a suppression of ACTH release follows administration of cortical hormones. Relatively little work has been reported, however, attempting to localize the site of this inhibitory action by cortical steroids. Earlier workers supposed that cortical hormones block the release of ACTH by an action on the adenohypophysis, itself. More recent work has focused attention on the role of the hypothalamus in the regulation of ACTH release (5) and, consequently, raised the possibility that the inhibitory action may be on central nervous structures.

In the present experiments two attempts have been made to localize the site of this inhibitory action of cortical steroids. In the first series of experiments rats with hypothalamic lesions designed to denervate the pituitary

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were employed, and the effect on ACTH release of lowering or elevating the cortical hormone titer of the blood was evaluated. A report of part of this work has already appeared (6). The second series of experiments was an attempt to study the effect of an excess of cortical hormones on the release of the hypothalamic neurohumor regulating ACTH secretion. Considerable evidence indicates that this neurohormone may be vasopressin (antidiuretic hormone (ADH)) (for review of the literature see (7, 8)). If this hypothesis is correct, evidence concerning the effect of cortical hormones on ADH release would be important in assigning a central nervous system site to the inhibitory action of cortical hormones. The results appear to indicate that cortical hormones suppress the release of ACTH both by suppression of the pituitary and by inhibition of the release of ADH.

METHODS

Male, Wistar rats weighing between 270 and 330 g. were used in all experiments. In the first series of experiments, rats with chronic hypothalamic lesions in the median eminence of the tuber cinereum were utilized (7, 9). Lesions were made with the Krieg stereotaxic instrument using coordinates which interrupt the supraoptico-hypophysial tract in the median eminence or just rostral to this structure, as determined by study of serial sections of a large series of brains. Although only a few of the brains from the present experiments were studied microscopically, in all cases an estimate of the degree of interruption of this tract was made by observation of increase in water intake of the animals. Experiments were begun 3–12 weeks after placement of lesions.

The basis for assessing alterations in ACTH output was the change in adrenal weight produced by the procedure in question. Using ether anesthesia, the left adrenal was removed first from each rat and weighed to the nearest 0.1 mg. on a torsion balance. Following this unilateral adrenalectomy several groups of rats were injected intramuscularly once a day with hydrocortisone free alcohol,[2] while others received no corticoid therapy. Two weeks after removal of the first gland the right adrenal was also removed for weighing. Because of variation in the initial adrenal weight, all results are expressed as the percentage change in weight of the right adrenal compared to the weight of the left.

In the second series of experiments, the antidiuretic response to emotional stress was compared in control and hydrocortisone-treated rats. The order of procedure was as follows:

1) Approximately four hours prior to exposure to stress, the hydrocortisone-treated group of rats was injected with 8 mg./100 g. of body weight of hydrocortisone acetate, intraperitoneally (I.P.). This dose completely blocks the ACTH release in response to severe, acute stress (10, 7).

2) Three to three and one-half hours after hydrocortisone injection, both the hydrocortisone-treated and the control rats were water-loaded by stomach tube with 5% of their body weight of 0.2% sodium chloride solution.

3) A second gavage was administered 60 minutes after the first in the case of the control rats. Because of their increased urine output, this second gavage was given 40 minutes after the first in the hydrocortisone-treated rats.

4) Urine was collected at 20 minute intervals, after prodding the animals to insure voiding. The stress was applied as soon after the 2nd gavage as the rat exhibited a

[2] We are indebted to Dr. A. Alpert, Merck-Sharp and Dohme, West Point, Pa. for generous supplies of hydrocortisone free alcohol.

diuresis of at least 3 ml./20 minute period, providing the water load was greater than 6% of the body weight.

5) The stress consisted of placing the rat on an electrified grid for 2 minutes immediately following the preceding urine collection. The grid was connected to the house AC current by means of a resistor, and the shock delivered was sufficient to cause considerable squeaking and jumping of the experimental animals.

In these experiments the water load of both control and hydrocortisone-treated rats was comparable. Since under these conditions, the steroid-treated rats had higher pre-shock urine flows than the controls, one series of experiments was performed in which rats treated with cortical hormone were given a smaller water load to make the control rate of urine excretion comparable to that of the normal rats.

Most rats were subjected to shock on 2 separate occasions; a few were shocked 4 times. The effect of shock on urine output was not altered by repeated tests. Half the animals were shocked first after steroid treatment, while the order of testing was reversed in the others. Shock was not repeated for at least a week after hydrocortisone injection, and no rat was subjected to experiment oftener than once every three days.

Lastly, in a final series of experiments the antidiuretic potency of plasma from normal and hydrocortisone-treated rats (8 mg. hydrocortisone acetate/100 g. body weight, I.P., 4 hours before testing) was assayed in water-loaded rats and compared to the activity of a Pitressin standard. In these experiments, plasma was obtained from etherized male rats by drawing 8–9 ml. of external jugular vein blood over a period of 2–4 minutes into a syringe containing 0.2 ml. of heparin. The blood was centrifuged in plastic tubes for 20–30 minutes, and the plasma injected I.P. into the assay rat at dose levels of 1 or 2 ml./100 g. body weight. The assay rats were water-loaded as described above except that no assay was performed unless the rat was excreting at least 1.2 ml./100 g. body weight of urine/20 minute period, and the water load was greater than 6% of the body weight. In some experiments a third gavage was administered before these requirements were met. All rats were accustomed to the gavage before use in assay of plasma or Pitressin and were used not oftener than once every three days.

RESULTS

1. *Alteration in the concentration of cortical hormones in the blood of rats with hypothalamic lesions.*

1) The effect of a reduction in cortical hormone concentration in the blood.

Unilateral adrenalectomy was performed to produce a reduction in the concentration of circulating adrenal cortical hormones, and the effect on ACTH release was measured by the percentage increase in the weight of the opposite (right) adrenal. Since no increase in weight occurs in unilaterally adrenalectomized, hypophysectomized rats, it is reasonable to ascribe any increase in weight of the opposite gland to an increase in ACTH release (11, 12). Twelve normal rats subjected to unilateral adrenalectomy responded with an increase of $51 \pm 8\%$ in right adrenal weight, when this gland was removed 2 weeks after the first. Fifty nine rats with hypothalamic lesions in the median eminence of the tuber cinereum showed a marked impairement in the response to unilateral adrenalectomy. When the percentage change in adrenal weight is compared with the water intake of the animals as an index of the severity of their diabetes insipidus (Fig.

1), a noticeable reduction in response is evident even when only a slight increase in water intake from the normal of 35 ml./day is present, the maximum effect being present when the water intake has increased to 80–100 ml./day. Increase in the severity of the diabetes insipidus to values up to 430 ml. water intake/day was not associated with any further decline in the response. The mean response of the 30 rats with water intakes greater than 100 ml./day was $14 \pm 3\%$, a value significantly less than the response of normal rats ($P < .001$). When both adrenals are removed simultaneously in rats with hypothalamic lesions, the right adrenal is $11 \pm 2\%$ smaller than the left; thus, the response of 14% in rats with lesions represents an actual

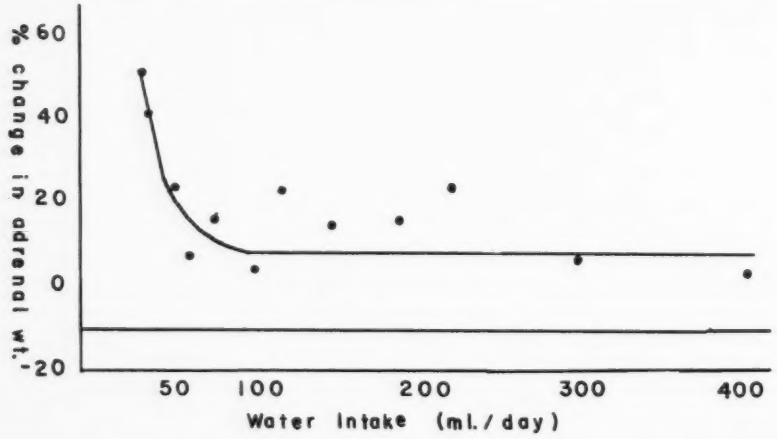

FIG. 1. Suppression of compensatory adrenal hypertrophy in rats with diabetes insipidus. Each point represents the mean response of 4 or more rats for each increment of 10 ml. or a multiple thereof of water consumption per day. The solid straight line is the result found in rats with lesions where both adrenals were removed simultaneously.

increase in weight of the right adrenal of 25%. This response is 40% as great as that exhibited by normal animals.

This failure of hypertrophy is similar to the reduction in resting adrenal weight, ascorbic acid depletion from stress, and blood ACTH concentration which has been reported previously in rats with diabetes insipidus (13, 14). All of these effects are obtained in rats with normal testicular weights implying a specific effect of these lesions on ACTH release. Only 10% of the rats of the present series had significant testicular atrophy.

Occasional rats appeared to show a complete failure of this hypertrophy; however, since one of 12 control animals also showed no compensatory hypertrophy, it is impossible to state that such animals, in fact, had any greater deficit in response than that portrayed by the mean. On plotting the percentage change in adrenal weight against the initial left adrenal weight, there was a suggestion that animals with an extremely small left

adrenal (<10 mg.) were unable to respond with any increase in adrenal weight. However, because of the small number of rats in this group (only 3), it is impossible to state that this would have always been the case. Thus, it is concluded that the release of ACTH following unilateral adrenalectomy is reduced but not completely prevented by hypothalamic lesions. On the basis of previous experience these same lesions would have been expected to completely block the response to severe, acute stress (13).

As an incidental finding it was noted that rats which had lost greater than 10% of their body weight, either in the period after placement of hypothalamic lesions and prior to unilateral adrenalectomy, or after unilateral adrenalectomy, gave a much more pronounced response to unilateral adrenalectomy. The weight loss appeared to be due to illness or simply to anorexia. Consequently, the results sighted above do not include animals of this type. The mean response of the 7 rats which lost greater than 10% of their body weight was $70 \pm 13\%$. This significantly greater gain in adrenal weight indicates that even rats with hypothalamic lesions can respond to chronic stress with an increased ACTH secretion.

The adrenal hypertrophy found in rats with very severe diabetes insipidus has previously been explained as being due to an increased ACTH release in response to the chronic stress of high water turnover (13, 14). A further study of the cause of this adrenal enlargement in rats with very severe diabetes insipidus has been made. Ten rats with severe diabetes insipidus (water intake = 300–340 ml./day) were treated for 2 weeks with a dose of Pitressin in oil (0.1–1.0 U., subcutaneously per day) sufficient to control the diabetes. After this treatment, the mean left adrenal weight of these rats was 18.4 ± 1.1 mg. The left adrenal weight of 37 untreated rats with diabetes insipidus of equal severity was 22.9 ± 0.9, a value which was significantly greater ($P < .02$) than that found in the rats where diabetes insipidus had been eliminated. Unilateral adrenalectomy with subsequent control of diabetes with Pitressin in oil was carried out in another group of 5 rats with diabetes insipidus (water intake = 200–600 ml./day) to see if elimination of the diabetes would alter the response to unilateral adrenalectomy. Whereas rats with uncontrolled diabetes insipidus gave an increase in right adrenal weight of $14 \pm 3\%$ in response to unilateral adrenalectomy, Pitressin-treated rats showed a decrease in right adrenal weight of $8 \pm 1\%$ as the response to the same procedure. Thus, the response to unilateral adrenalectomy is significantly less ($P < .001$) after control of the diabetes with Pitressin. The smaller adrenals found after control of diabetes and the diminished response to unilateral adrenalectomy of Pitressin-treated rats are interpreted to mean that the ACTH secretion of the rat with diabetes is reduced by elimination of the high water turnover. Therefore, it seems reasonable to attribute the adrenal hypertrophy found in rats with severe, uncontrolled diabetes insipidus to the stress of high water turnover, which produces an increased secretion of ACTH even in the

presence of an effective hypothalamic lesion. It is apparent from these data that not only is there a residual response to a decrease in the concentration of cortical hormones in rats with hypothalamic lesions, but also that these rats can show responses to chronic stress stimuli, such as infection, inanition, and the high water turnover of diabetes insipidus.

2) The effect of an increase in cortical hormone concentration of the blood.

In these experiments elevations in the blood level of cortical hormone were produced by daily intramuscular injections of hydrocortisone free

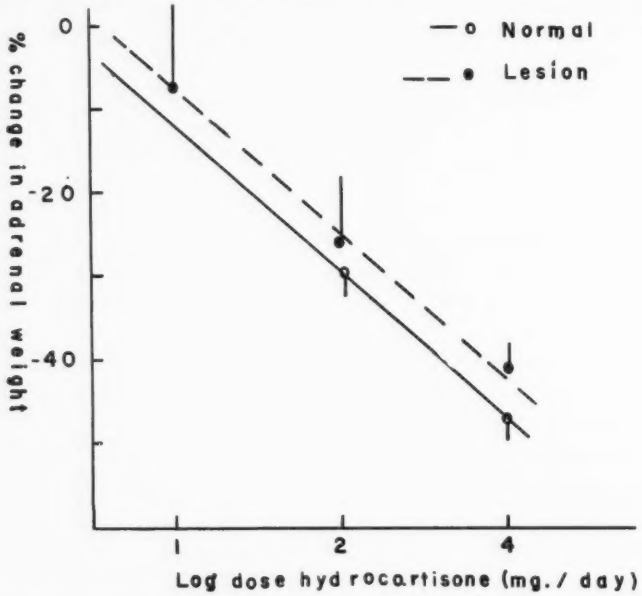

FIG. 2. The effect of hydrocortisone injections on adrenal weight of normal rats and those with hypothalamic lesions. The vertical lines represent the standard error.

alcohol. This is one of the steroids produced by the rat adrenal cortex, though the main component of the secretion appears to be corticosterone (15). Presumably, administration of cortical hormone should obviate the need for increased ACTH secretion in the unilaterally adrenalectomized rat and result in a decrease in compensatory hypertrophy or even in compensatory atrophy of the gland as the dose of steroid is increased.

The results of investigation of 3 different dosages of hydrocortisone in unilaterally adrenalectomized normal rats and those with lesions are depicted in Figure 2. Only rats with lesions which were drinking more than 100 ml. of water/day were used since these animals have a uniformly depressed response to unilateral adrenalectomy per se. It can be seen that following steroid treatment the mean response of both normal rats and

those with lesions was indistinguishable, and that in both cases the results lay on the same log dose response curve (Fig. 2). A dose of 1 mg./day appeared to be just sufficient to block compensatory hypertrophy completely, whereas 4 mg./day produced near maximal adrenal atrophy.

When the percentage change in right adrenal weight of the rats with hypothalamic lesions is plotted versus their water intake (Fig. 3), it is apparent that the dose of 1 mg./day induced adrenal atrophy only in animals with mild diabetes insipidus; 2 mg./day induced a reduction in adrenal weight in all rats except those with the most severe diabetes insipidus; whereas 4 mg./day produced adrenal atrophy in all rats regardless of the severity of the diabetes insipidus.

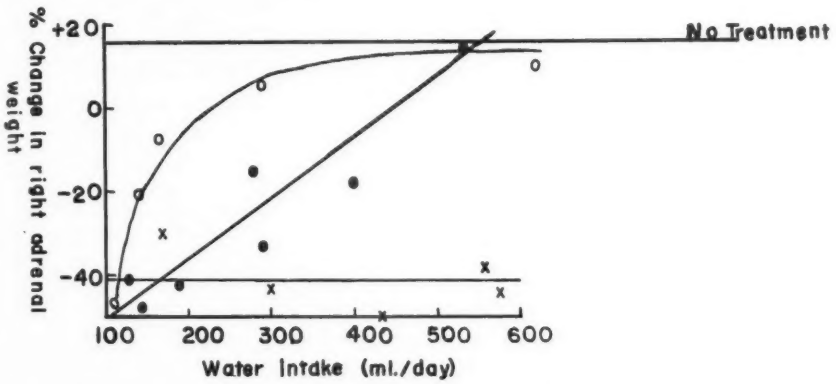

FIG. 3. The effect of hydrocortisone on adrenal weight of rats with lesions and varying severity of diabetes insipidus. Dose: 0.1 mg./day, 0.2 mg./day, ×4 mg./day.

The results, therefore, clearly show that rats with hypothalamic lesions respond to elevation of cortical hormone level with a reduction in adrenal weight indicative of a suppression of ACTH secretion. Whereas rats with mild diabetes insipidus appear to require less hormone to accomplish this effect than normal rats, those with severe diabetes insipidus require more than normal rats.

2. *The effect of elevation in the blood cortical hormone concentration on the discharge of ADH*

1) The effect of hydrocortisone on the antidiuresis induced by emotional stress.

Before considering the effects of stress on urine flow, it is important to note that equivalent water loads result in a greater diurseis in steroid-treated rats than that obtained in normals (Fig. 4) as reported earlier by others (16). The mean pre-stress water excretion in the hydrocortisone-treated rats was 6.9 ml./20 minutes while the control rats excreted 4.4 ml./20 minutes. Because of variation in pre-stress urine flows, particularly

in the steroid-treated rats, the decrease in urine flow produced by stress was also quite variable. To reduce this variability we have chosen to express the results as percentage decrease in urine flow in response to stress.[3]

The effects of the stress of electric shock on urine flow are depicted in Figure 4. Table 1 summarizes the results expressed in terms of the percentage decrease in urine flow, while Table 2 presents the data from all rats where shock was administered to the same rat both with and without hydrocortisone therapy. It can be seen in Table 1 that the rate of urine flow remained constant during the experimental period if no shock was given. Both normal and hydrocortisone-treated rats responded to the

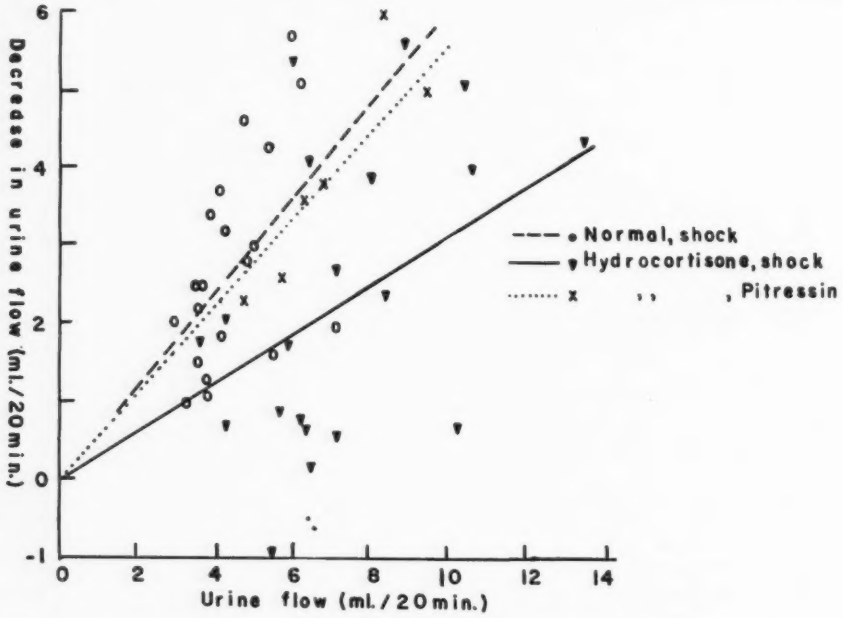

FIG. 4. The antidiuretic effect of stress on urine flow of normal rats and rats treated with hydrocortisone.

stress of electric shock with a reduction in urine flow. The mean percentage decrease of urine flow in the normals was $62 \pm 5\%$, while the decrease in the steroid-treated rats was only $32 \pm 6\%$. Thus, the antidiuresis induced by shock was only half as great in the steroid-treated rats as in the normals. The results were similar in the nine experiments where the water

[3] This is justifiable statistically since: 1) there is a significant correlation between the reduction in urine output and the pre-stress urine flow (r combined = 0.547, $P < .01$) (Fig. 5); 2) the regression lines obtained on plotting decrease in urine flow (y) versus prestress urine flow (x) pass through the origin (Fig. 5); and 3) the standard deviation of y increases with increasing values of x (17).

TABLE 1. RESPONSE TO SHOCK AND VASOPRESSIN OF NORMAL AND HYDROCORTISONE-TREATED RATS

Treatment	% decrease in urine output during 20 minutes post-treatment	
	Normal	Hydrocortisone-treated
No Treatment	-2 ± 6[1] (6)[2]	-1 ± 8 (7)
Shock	62 ± 5 (20)	32 ± 6 (20)
Pitressin, 2 mu., I.P.	57 ± 8 (7)	56 ± 4 (6)

[1] Standard error of mean.
[2] No. of rats per group.

load of the steroid-treated rats was decreased to make the initial urine flow comparable to that seen in normal rats. Furthermore, the response at all levels of urine flow in the treated rats was similar. Thus, the results cannot be ascribed to the greater initial urine flow exhibited by many of the steroid-treated rats and indicate that stress-induced antidiuresis is attenuated by steroid treatment.

The attenuated antidiuresis of the hydrocortisone-treated rat could not be attributed to an alteration in sensitivity to ADH since the I.P. injection of 2 mu. of Pitressin to both groups of rats was followed by identical percentage decreases in urine output (Table 1). Similarly, these later results make it unlikely that alterations in disposal rate of ADH contribute to the effect. Since the reactivity to the shock stimulus itself, as evidenced by squeaking and jumping, was not altered, it was concluded that the antidiuretic response to shock of hydrocortisone-treated rats is reduced. This reduction could be due either to alterations in renal haemodynamics, or to a reduction in the release of ADH from the neurohypophysis.

2) The effect of hydrocortisone on the ADH concentration of the plasma of stressed rats.

TABLE 2. COMPARISON OF THE EFFECT OF SHOCK WITH OR WITHOUT HYDROCORTISONE TREATMENT IN THE SAME RAT

% decrease in urine flow		
No treatment	Hydrocortisone	Difference
88	40	48
81	6	75
76	68	8
28	11	17
69	40	29
89	38	51
69	8	61
60	38	22
78	16	62
73	64	9
98	46	52
29	−16	45
34	31	3
58	13	45
Mean = 66	29	37 ± 6[1]

[1] Standard error of mean; $P < .001$.

TABLE 3.—ANTIDIURETIC POTENCY OF PLASMA

Source	No. of Assay Rats	Vol. of Plasma Injected (ml./100 g.)	% Decrease in Urine Flow (Mean ± S.E.)	Potency (mU. pitressin/100 ml.)
1) Jugular plasma from stressed rats with diabetes insipidus	7	2	5 ± 6	< 1
2) Jugular plasma from stressed, normal rats	20	2	64 ± 4	15 ± 3
		1	43 ± 6	15 ± 3
3) Jugular plasma from stressed rats treated with hydrocortisone	20	2	42 ± 8	7 ± 2
		1	31 ± 6	
4) Plasma from rats decapitated under Nembutal	14	2	20 ± 5	3 ± 1

The ADH titer of plasma was assayed in water-loaded rats. The results were expressed as percentage decrease in urine flow (18) over a 40 minute period post-injection. Control I.P. injections of 0.5 or 2 ml. of isotonic saline produced no effect on the urine flow of the assay rats. The results of assay of plasma are presented in Tables 3 and 4, and the standard curve for Pitressin in Figure 5. It can be seen that under our particular experi-

TABLE 4. ASSAY OF JUGULAR VEIN PLASMA OF NORMAL AND HYDROCORTISONE-TREATED RATS UNDER STRESS

Vol. Injected/100 g. rat	Normal donors		Hydrocortisone-treated donors	
	1 ml.	2 ml.	1 ml.	2 ml.
Response (Percentage decrease in urine flow for 40 minutes post injection)	27	30	38	71
	42	69	25	56
	52	50	11	10
	15	69	16	61
	48	82	14	47
	67	72	58	69
	38	56	52	43
	53	57	46	−13
		70		67
		77		58
		74		24
		62		15
Mean	43 ± 6	64 ± 4	31 ± 6	42 ± 8
Potency in mU. of pitressin/100 ml.	15 ± 3		7 ± 2	

$$\text{Log Potency } \frac{\text{Treated Plasma}}{\text{Control Plasma}} = -.328 \pm .116$$

$$\text{Potency } \frac{\text{Treated Plasma}}{\text{Control Plasma}} = 47 \pm 15\%^{1}$$

95% Confidence limits = 27–81%
$b_c = 59.4$; $s_c = 20$; $\lambda_c = .37$

[1] Calculated by method of Stein, Jinks, and Mirsky (1952), the respective values were:

$$\text{potency } \frac{\text{treated plasma}}{\text{control plasma}} = 53 \pm 13\%,$$

potency of control plasma in mU. Pitressin/100 = 14 ± 3; potency of hydrocortisone treated plasma = 7 ± 2.

mental conditions plasma from rats with diabetes insipidus[4] contained no detectable ADH (<1 mU. Pitressin/100 ml. plasma). Consequently, the antidiuresis evoked by other plasma samples can be attributed to the content of ADH in the samples. Plasma from rats decapitated 5 minutes after initiation of Nembutal anesthesia (4 mg./100 g., I.P.) contained appreciable, though barely detectable levels of ADH (3 ± 1 mU./100 ml.). This titer appeared to be too low to use in evaluating the effects of hydrocortisone treatment. Ginsburg and Heller (18) have reported particularly high

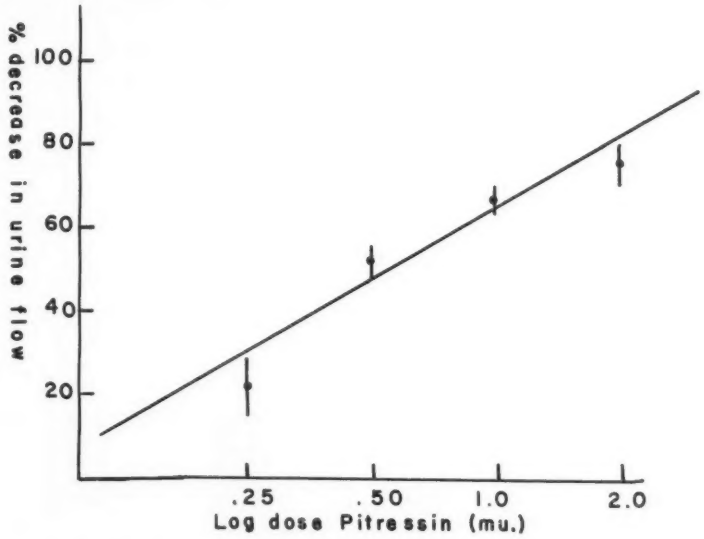

FIG. 5. The log-dose response curve of Pitressin in hydrated rats. The vertical lines represent the standard error.

titers of ADH under the combined stress of ether anesthesia and exsanguination and have also shown that the concentration of ADH is higher in external jugular blood of the rat than in arterial blood. Therefore, we chose to test the effect of hydrocortisone on the ADH titer of external jugular vein plasma taken from etherized, exsanguinated rats.

The results of assay of jugular plasma from hydrocortisone-treated and normal rats are shown in Table 4. Plasma from steroid-treated rats was $47 \pm 15\%$ as potent as normal plasma and contained 7 ± 2 mU./100 ml. compared to 15 ± 3 mU./100 ml. Pitressin equivalent. Thus, these results coupled with those obtained in shocked rats indicate that though hydrocortisone fails to completely block the release of ADH from the neurohypophysis in response to stress, it does produce a 50% reduction in this release.

[4] This diabetes insipidus was produced by lesions in the supraoptico-hypophysial tract the preceding day; the water intake was greater than 100 ml./day.

DISCUSSION

The results obtained in the rats with lesions are summarized in Figure 6 and have been interpreted as follows: rats with effective hypothalamic lesions have a reduced sensitivity to alterations in the concentration of circulating corticoids; this is supported by the findings with unilateral adrenalectomy, where a 50% reduction in concentration of cortical hormones resulted in only a small response. If the concentration of hormone is elevated sufficiently above the basal level, suppression of ACTH release with a consequent adrenal atrophy is the response even in rats with lesions.

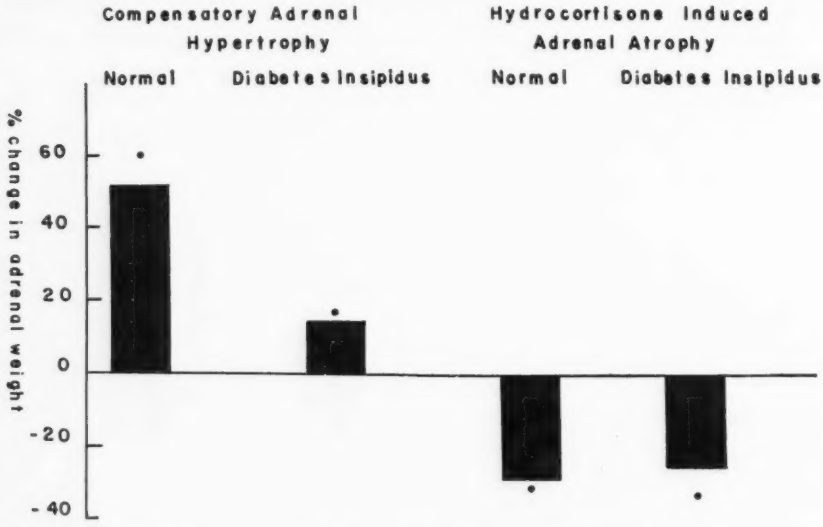

FIG. 6. Summarization of the effects of altered cortical hormone levels on adrenal weight.

Since animals with mild diabetes insipidus required smaller doses of corticoids to produce atrophy than normal rats or those with severe diabetes insipidus, it is postulated that the basal concentration of cortical hormones in mildly diabetic rats is lower than that found in the other two groups. Accordingly, a smaller dose of cortical hormone would produce a greater increment in corticoid titer in mildly diabetic rats than in either of the other two groups and shut down the release of ACTH. This interpretation is consistent with the observed adrenal atrophy in rats with mild diabetes insipidus (13), which also suggests a decreased basal output of corticoids. Rats with severe diabetes insipidus required larger doses of cortical hormone to suppress ACTH secretion than rats with mild diabetes or normals, presumably because of the increased blood corticoid concentration present in these animals as a response to the stress of diabetes insipidus. Since alterations in cortical hormones can still act in the presence

of hypothalamic lesions, which presumably denervate the pituitary, the conclusion is almost inescapable that these hormones must act on a site other than the central nervous system in this situation, presumably the anterior lobe, itself.

Recently Rose and Nelson (19) have reported effects of local injections of corticoids into the pituitary which are consistent with a suppressive effect of target gland hormones on pituitary ACTH secretion. Ganong and Hume (20, 21) have made observations on dogs with hypothalamic lesions which are in substantial agreement with those reported here except that they found no response to unilateral adrenalectomy whatsoever, whereas our results indicate that an attenuated response persists in the rat.

Since these rats with hypothalamic lesions which block the acute release of ACTH in response to stress (13) respond to chronic stress, the denervated pituitary is pictured as producing a decreased basal secretion of ACTH resulting in adrenal atrophy and a subnormal secretion of adrenal steroids. It is insensitive to acute stress stimuli but is still capable of responding sluggishly to chronic stress or alteration in cortical hormone titer. Whether chronic stress acts via changes in corticoid titer produced as a result of stress-induced alterations in utilization rate of steroids or through some unidentified humoral mechanism is an enigma.

The results obtained with hydrocortisone over dosage indicate that a rise in cortical hormone titer of the blood can partially inhibit the release of ADH by the supraoptico-hypophysial system. Whether this action is exerted directly on cells of the supraoptic nucleus or at some other site which in turn affects the excitation of these cells cannot be stated from the present results. Earlier results indicating that diabetes insipidus-like conditions can be produced by cortical hormones (16) and that chronic administration of hydrocortisone produces an abnormal rise in posterior lobe ADH content in response to stress (22) are consistent with a suppressive action of elevated corticoid levels on ADH release. Likewise, the delay in water diuresis and elevated ADH titer reported by some workers in adrenalectomized rats (23, 24) would fit with a concept of an inverse relation between ADH secretion on the one hand and cortical hormone titer on the other. Thus, if ADH is the neurohumor stimulating the release of ACTH, the suppressive action of cortical hormones on ADH release could explain, in part at least, their effect in inhibiting ACTH release. Our interpretation would be that cortical hormones suppress ACTH release by a dual action, suppressing both the release of the hypothalamic neurohumor and the excitability of the adenohypophysis, itself.

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PATHOLOGIC CHANGES IN SENSITIZED RATS TREATED WITH METHYLANDROSTENEDIOL AND WITH GROWTH HORMONE[1]

W. A. J. CRANE, R. E. PORTER AND D. J. INGLE

The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Illinois

ABSTRACT

The chronic administration of large doses of methylandrostenediol (MAD) to the sensitized rat (unilateral nephrectomy and a 4% sodium chloride diet) causes hypertension, renal and cardiovascular pathology. In the present study MAD was administered to non-adrenalectomized, sensitized rats and to adrenalectomized-sensitized rats treated with adrenal cortex extract (ACE). The control groups did not receive MAD. Experiments 1 and 2 involved rats eating *ad libitum*. The dose of MAD was 10 mg. per rat per day for 56 days; the adrenalectomized rats were each given 2 cc. of ACE per day. The rats given MAD developed hypertension and renal and cardiovascular pathology in both the presence and absence of the adrenal glands. In Experiment 3, force-fed rats were given 20 mg. of MAD per rat per day; the adrenalectomized rats were each given 1 cc. of ACE per day. The duration of the experiment was 42 days. Hypertension and renal and cardiovascular changes were more severe in the non-adrenalectomized rats. It is concluded that the damaging effects of large doses of MAD can occur in the absence of the adrenal glands but that the presence of adrenal cortical hormones is required.

In Experiment 4, a highly active preparation of growth hormone was given to sensitized rats eating *ad libitum*. There was an increase in body weight, food intake and all of the rats given GH developed a polydipsia, but the elevation in blood pressure and renal and cardiovascular changes were minimal.

IT WAS discovered by Skelton (1) that the chronic administration of large doses of methylandrostenediol (MAD) to the sensitized (unilateral nephrectomy and a high sodium chloride intake) rat causes hypertension, renal, and myocardial pathology. Subsequently Salgado and Selye (2) reported that whereas MAD causes a syndrome of hyalinosis, with hypertension and polyuria in the sensitized rat having intact adrenals, it does not do so in the adrenalectomized-sensitized rat.

The data of the present study show that MAD can cause hypertension with renal and myocardial pathology in the adrenalectomized-sensitized rat treated with "normalizing" doses of adrenal cortex extract.

An additional experiment involved the administration of growth-hormone to the sensitized rat. The damage was minimal.

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METHODS

Male rats of the Sprague-Dawley strain were maintained on Archer Dog Pellets until they reached a weight of approximately 300 grams. They were then adapted to either the *ad libitum* eating of a dry high sodium chloride diet (Table 1) or to the tube feeding of the same diet made fluid by the addition of water. When the dry diet was used it was tightly packed into individual Franke feeding jars which were weighed every 24 hours. Other animals were adapted to the tube-feeding of 13 cc. of fluid diet each morning and late afternoon.

Either a unilateral nephrectomy only, or unilateral nephrectomy plus bilateral adrenalectomy was done in a single stage operation. Clean but not sterile technique was used. Immediately following operation each rat was given 5000 units of penicillin and 5 mg. of streptomycin. This was not continued.

Methylandrostenediol (Ciba, Protandren) was given as an aqueous suspension of microcrystals by subcutaneous injection twice daily. Beef adrenal cortex extract (Upjohn) was given in aqueous solution (ethanol-free) by subcutaneous injection twice daily. Growth hormone (GH) prepared by the Armour Laboratories (Somar-A) was supplied by the Endocrinology Study Section of the National Institutes of Health. It was made up in aqueous solution and administered by subcutaneous injection twice daily.

TABLE 1. HIGH SALT MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour	60
Salt mixture U.S.P. XIV	40
Dried yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Mazola oil plus 100 mg. K	10
Mazola oil	190
Casein	160
Starch	300
Dextrin	150
Sucrose	140
Sodium chloride	50
To make fluid diet add water	1040

For the determination of blood-pressure the rats were anesthetized with ether and the abdominal aorta was cannulated below the level of the renal arteries. The cannula was attached by a short length of fine-bore rubber tube filled with heparinized saline to a mercury manometer. The record was taken when anesthesia was light as judged by retraction of the rat's hind foot on pinching.

All rats were killed by exsanguination under ether anesthesia at the end of either 8 weeks or at 6 weeks in one experiment. Autopsy was performed and gross pathologic changes noted. The heart, kidney and portions of liver, spleen, pancreas, lung, stomach and intestine were fixed in 10% neutral formalin. Hematoxylin and eosin was the routine stain and selected examples of heart and kidney were examined by the periodic acid-Schiff routine, and Weigert's resorcin-fuchsin stain for elastic tissue. Blocks of kidney were embedded in gelatin and frozen sections were examined for lipid with Oil Red O (3) and for cholesterol by the Schultz method (4). One adrenal gland was fixed in 10% neutral formalin and the other in cold acetone. Paraffin sections were stained with hematoxylin and eosin and with the periodic acid-Schiff method. Frozen sections of gelatin embedded adrenals were examined with Oil Red O for lipids (3), the Schultz method for cholesterol (4), and with polarized light for birefringent crystals. Acetone processed material was used for the Gomori alkaline phosphatase procedure (3).

EXPERIMENTS AND RESULTS

Experiment 1 (Table 2) involved non-adrenalectomized sensitized rats eating *ad libitum;* 7 rats served as untreated controls and 7 rats each received 10 mg. of MAD per day for 56 days.

Experiment 2 (Table 2) involved 30 sensitized rats eating *ad libitum,* divided into 4 groups as follows: 7 rats with adrenals intact were untreated; 7 rats with adrenals intact each received 10 mg. of MAD per day; 8 adrenalectomized rats were each given 2 cc. of ACE per day without MAD; and 8 adrenalectomized rats were each given 2 cc. of ACE and 10 mg. of MAD per day. The duration of the experiment was 56 days.

Experiment 3 (Table 2) involved 16 force-fed sensitized rats divided into 4 groups as follows: 4 rats with adrenals intact were untreated; 4 rats with adrenals intact each received 20 mg. of MAD per day; 4 adrenalectomized rats were each given 1 cc. of ACE per day without MAD; and 4 adrenalectomized rats were each given 1 cc. of ACE with 20 mg. of MAD per day. This experiment was terminated at 42 days because some of the rats, both with and without adrenal glands, were overtly ill due to MAD-toxicity.

Experiment 4 (Table 3) involved 14 non-adrenalectomized, sensitized rats eating *ad libitum;* 7 rats served as untreated controls and 7 rats each received 2 mg. of GH by subcutaneous injection each day for 56 days.

Food intake. There was a significant suppression of food intake in all of the rats given MAD. The rats given growth hormone ate more food than their controls and developed a polydipsia; during the last two days of the experiment the water intake of the controls averaged 55 cc. per 24 hours per rat and the rats treated with GH averaged 97 cc. per 24 hours per rat.

Body weight. The rats given the large doses of MAD gained less weight than the controls. This was not due solely to suppression of appetite, since the force-fed rats of Experiment 3 showed some suppression of weight gains among the rats given MAD. The rats given GH gained much more weight than did the controls.

Other gross changes. All of the rats given MAD showed inhibition of hair growth, more marked in the rats having intact adrenals than in the adrenalectomized rats treated with ACE. All of the rats in the control groups showed apparently normal hair growth. Most of the rats given MAD developed edema of skin and subcutaneous tissues. This was commonly present when the rats were observed in the early morning and would then disappear during the day. It was sometimes so marked as to form pendulous sacs of fluid. This was not seen in any of the control animals. The observers were left with the impression that large doses of MAD have an extra-adrenal effect upon salt and water metabolism. Gonadal atrophy with hypertrophy of the seminal vesicles and prostate was seen in all rats given MAD.

Blood-pressure. The average pressure of 12 normal rats was 121 ± 2.9.

TABLE 2. EFFECTS OF METHYLANDROSTENEDIOL (MAD) ON THE SENSITIZED RAT IN THE PRESENCE AND ABSENCE OF THE ADRENAL GLANDS. AVERAGES, STANDARD ERRORS, AND RANGE

Experimental conditions	N rats	Body-weight, gm.		Organ weights, mg.				Mm.Hg BP	Gm. food daily	Ratings of pathology, average and range*		
		Initial	Final	Heart	Kidney	Thymus	One adrenal			Kidney		Heart, micro
										Gross	Micro	
Adrenals intact, no MAD 56 days. Eat *ad lib.*	7	347	446 ±10.7	1343 ±51.0	2031 ±53.3	411 ±36.9	29.3 ±0.9	134 ± 3.2	17.7	1.2±0.02 (0.5–2.0)	0.8±0.10 (0.0–2.0)	0.3±0.39 (0.0–2.0)
Adrenals intact, 10 mg. MAD daily. 56 days. Eat *ad lib.*	7	351	357 ± 5.7	1367 ±52.7	4518 ±590	38 ± 2.7	35.2 ± 2.1	167 ±10.1	15.7	3.2 ±0.59 (1.0-5.0)	3.9±0.60 (1.0-50)	1.9±0.10 (0.0-30)
Adrenals intact, no MAD. 56 days. Eat *ad lib.*	7	339 ±4.2	450 ± 9.9	1320 ±40.6	2146 ±63.5	406 ±41.6	28.3 ± 1.7	139 ± 3.9	18.6	0.93±0.13 (0.5–1.5)	1.2±0.26 (0.5–2.0)	1.1±0.40 (0.0–2.0)
Adrenals intact, 10 mg. MAD daily. 56 days. Eat *ad lib.*	7	338 ±4.1	346 ± 5.7	1284 ±32.4	4290 ±321	33 ± 5.0	29.9 ± 0.9	164 ± 8.2	13.8	2.6 ±0.43 (1.0–4.0)	3.6±0.37 (3.0–5.0)	2.3±0.40 (0.0–3.0)
Adrenalectomy, 2 cc. ACE daily, no MAD. 56 days. Eat *ad lib.*	8	335 ±5.0	431 ±10.4	1376 ±36.4	2241 ±109	421 ±45.7		152 ± 3.6	16.6	0.8 ±0.16 (0.0±1.5)	1.2±0.39 (0.5–3.0)	1.0±0.38 (0.0–2.0)
Adrenalectomy, 2 cc ACE plus 10 mg. MAD daily. 56 days. Eat *ad lib.*	8	334 ±4.6	363 ± 9.8	1306 ±45.1	4386 ±413	43 ± 4.7		166 ± 6.6	13.8	2.9± 0.55 (1.0–5.0)	3.5±0.32 (3.0–5.0)	1.9±0.30 (0.0–3.0)
Adrenals intact, no MAD. 42 days. Force-feed.	4	304 ±2.1	366 ± 4.3	1060 ±38.9	1570 ±49.5	331 ±11.1	25.1 ± 1.5	133 ± 2.8	15.9	1.25±0.25 (1.0–2.0)	1.4±0.55 (0.5–3.0)	0.5±0.5 (0.0–2.0)
Adrenals intact. 20 mg. MAD daily, 42 days. Force-feed.	4	306 ±3.5	359 ± 6.0	1411 ±83.5	4394 ±308	38 ± 4.5	36.5 ± 0.65	169 ± 6.2	15.9	4.0 ±0.61 (2.5–5.0)	5.0±0.0 (5.0–5.0)	4.0±0.0 (4.0–4.0)
Adrenalectomy, 1 cc. ACE daily, no MAD. 42 days. Force-feed.	4	305 ±2.9	389 ± 4.1	1152 ±45.9	1790 ±20.4	610 ±38.2		139 ± 1.5	15.9	0.63±0.12 (0.5–1.0)	1.8±0.48 (1.0–3.0)	0.5± .05) (0.0–2.0
Adrenalectomy, 1 cc. ACE plus 20 mg. MAD daily. 42 days. Force-feed.	4	307 ±2.7	371 ± 4.1	1260 ±34.3	4130 ±124	36 ± 3.9		151 ±11.1	15.9	1.6 ±0.38 (1.0–2.5)	3.5±0.5 (3.0–5.0)	1.3±0.7 (0.0–3.0)

* The ratings of gross renal pathology are based on the following scale: 0 = no abnormality; 1 = few tiny nodules; 2 = moderately nodular with some mottling; 3 = more nodules, mottling with some gray spots; 4 = rough, mottled, with gray and red spots; and 5 = changes listed previously but more severe, giving "flea-bitten" grayish-yellow appearance.

The ratings of the microscopic changes are based upon the following features.

Kidney: 0 = no abnormality; 1 = hypertrophy of glomeruli and tubules, a few hyaline casts, and slight focal interstitial inflammation; 2 = active pelvic inflammation; 3 = glomerular lesions, no apparent vascular damage; 4 = hyalinization of glomeruli, sclerosis of arterioles; and 5 = necrosis of glomeruli and arterioles.

Heart: 0 = no abnormality; 1 = hypertrophy of muscle; 2 = focal inflammation or fibrosis of myocardium; 3 = changes listed previously, sclerosis of vessel walls; and 4 = necrosis of myocardium and/or vessel walls.

TABLE 3. EFFECTS OF GROWTH HORMONE ON THE SENSITIZED RAT. AVERAGES, STANDARD ERRORS, AND RANGE

Experimental conditions	N rats	Body-weight, gm.		Organ weights, mg.				Mm. Hg BP	Gm. food daily	Kidney*		Heart,* micro
		Initial	Final	Heart	Kidney	Thymus	One adrenal			Gross	Micro	
Controls, 56 days	7	306 ±1.61	409 ±11.8	1134 ±33.0	1869 ±85.5	441 ±42.9	22 ±0.9	135 ±2.9	15.7	0.71 ±0.1	0.86	0
GH 2 mg./rat/day, 56 days	7	308 ±1.11	474 ±8.9	1454 ±54.7	2033 ±94.7	458 ±17.0	37 ±2.5	145 ±3.1	18.4	1.28 ±0.3	1.28 ±0.45	0

* The ratings of gross renal pathology are based on the following scale: 0 = no abnormality; 1 = few tiny nodules; 2 = moderately nodular with some mottling; 3 = more nodules, mottling with some gray spots; 4 = rough, mottled, with gray and red spots; and 5 = changes listed previously, more severe giving "flea-bitten" grayish-yellow appearance.

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Heart: 0 = no abnormality; 1 = hypertrophy of muscle; 2 = focal inflammation or fibrosis of myocardium; 3 = changes listed previously, sclerosis of vessel walls; and 4 = necrosis of myocardium and/or vessel walls.

The average pressure of control sensitized rats is higher than normal. Treatment of the adrenalectomized-sensitized rat with 2 cc. of ACE per rat per day is associated with a higher average pressure than is found in non-adrenalectomized controls. The administration of MAD is followed by a significant rise in pressure in non-adrenalectomized sensitized rats and in adrenalectomized-sensitized rats treated with ACE. The administration of growth hormone was associated with a small average rise in pressure.

Heart. The heart was relatively enlarged in proportion to body weight in the rats given MAD. The rats given 20 mg. of MAD daily showed an absolute gain in heart weight. A few of the rats given MAD showed white patches at either the apex or on the walls of the heart. No gross damage was seen in any heart from rats given growth hormone.

The cardiac changes in all rats were evaluated microscopically (footnote, Table 2) depending on the presence of cardiac hypertrophy (Grade 1), focal inflammation or fibrosis of the myocardium (Grade 2) and the association of these changes with hypertensive vascular sclerosis (Grade 3) and hypertensive arteriolonecrosis (Grade 4). The average ratings of damage for the control rats, both the intact and adrenalectomized-ACE treated groups, were comparable in any one experiment but varied slightly between individual experiments (Table 2). The majority of these hearts were normal, but a few showed granulomatous nodules composed of macrophages, myocytes, and fibroblasts. It is of interest that examples of this damage occurred in sensitized rats without hormonal treatment.

With MAD administration the average ratings and the range of damage was increased in both the intact rats and in the adrenalectomized rats treated with ACE (Table 2). Some hypertrophy of muscle cells occurred in response to hypertension. The incidence of granulomatous foci in the myocardium was increased and in both groups the PAS stain demonstrated "colloid granules" in the nodules as described by Salgado and Selye (2). The significance of this finding is not clear, since in our experiments granules of similar staining reaction were seen in some control rats not treated with MAD. Hypertensive vascular damage involved the coronary system of both the intact and adrenalectomized rats treated with ACE following MAD administration at both dose levels used in experiments 2 and 3 (Table 2). Proliferative arteriolosclerosis was present in intact and adrenalectomized rats treated with 10 mg. MAD and in adrenalectomized rats receiving 20 mg. MAD (Fig. 1). With the higher dose of MAD in intact rats (Experiment 3) the arteriolar walls were necrotic. Both types of vascular damage were associated with replacement fibrosis of the myocardium and in those hearts which showed arteriolonecrosis actual necrosis of cardiac muscle cells resulted (Fig. 2).

No damage was seen microscopically in any heart from rats treated with GH (Table 3).

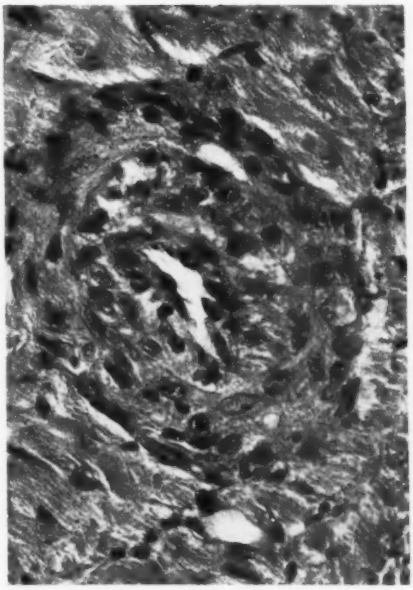

FIG. 1. Heart. Adrenx. rat, MAD 10 mg. +ACE 2 cc. Intimal thickening of a small coronary artery. BP 192 mm. Hg. Hematoxylin and eosin ×795.

FIG. 2. Heart. Intact rat, MAD 20 mg. Myocardial fibrosis and arterial hypertrophy. BP 157 mm. Hg. H and E ×258.

Kidney. The renal changes in all rats were evaluated as indicated in the footnote to Table 2. Microscopically minimal renal damage consisted of focal areas of periglomerular fibrosis with some tubular casts and round cell infiltration (Grade 1). It was considered that this was probably the result of focal chronic inflammation and in some cases it was associated with chronic inflammation of the renal pelvis (Grade 2). Examples of these types of damage were seen in control animals as well as those treated with MAD. Glomerular tuft lesions (Grade 3) associated with renal vascular damage (Grades 4 and 5) were characteristic of MAD treatment.

MAD caused a striking increase in the weight of the kidney in both intact rats and in adrenalectomized rats treated with ACE as compared with their respective controls (Table 2). These large kidneys had an unusual yellowish-green pallor. Frequently the surface showed numerous tiny cystic nodules with intervening pits and a few grey or hemorrhagic spots. The degree of gross renal damage corresponded closely to the microscopic assessment (Footnote and Table 2).

The average ratings and range of renal damage were identical when 10 mg. of MAD was administered to intact rats and to adrenalectomized rats treated with ACE (Experiment 2). With higher and lower doses of MAD and ACE respectively (Experiment 3) the average rating in adrenal-

FIG. 3. Kidney. Intact rat. MAD 10 mg. Lipid (black) is present in the hypertrophied afferent arteriole (top), in the glomerulus and related tubules. BP 177 mm. Hg. Oil Red O and hematoxylin ×345.

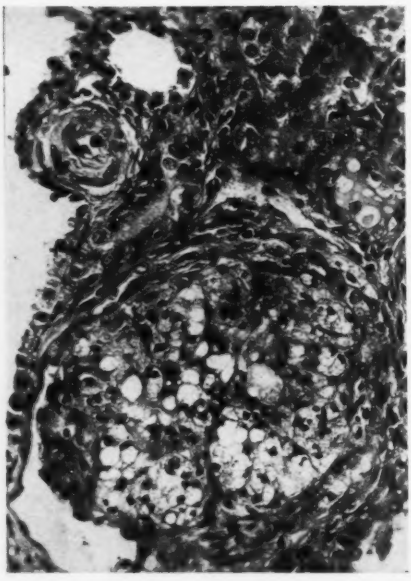

FIG. 4. Kidney. Intact rat, MAD 10 mg. The glomerulus is enlarged and adherent to the thickened capsule. Many of the cells show a "foamy" appearance. Hypertensive sclerosis of afferent arteriole (top left). BP 187 mm. Hg. H and E ×475.

ectomized rats was reduced as compared with the intact rats but the range of damage within the groups was comparable (Table 2). All rats which received MAD demonstrated the renotropic effect of this steroid irrespective of the severity of the hypertensive damage to the kidney. Enlargement of individual glomeruli varied. The largest showed an unusual appearance of swelling of the visceral epithelium of the glomerular tuft. The cytoplasm of these cells had a "ground glass" appearance and in some, but not all, affected glomeruli intracellular lipid and cholesterol were demonstrated (Fig. 3). Similar material was present in the epithelium of the related cortical tubules. The hypertensive nephrosclerotic effects of MAD were seen both in intact rats and in adrenalectomized rats treated with ACE. Proliferative arteriolosclerosis and arteriolonecrosis of the afferent arteriole were associated with damage to the glomerulus which varied from early hyalinization to advanced glomerulonecrosis (Figs. 4 and 5). Rarely the larger renal arteries showed lesions bearing some of the characteristics of polyarteritis nodosa (Fig. 6).

The kidneys from rats treated with GH were not enlarged relative to body weight (Table 3). Compared with the controls, the increase in gross renal damage was slight and significant lesions were minimal microscopically. Only two rats showed scant focal glomerular damage consisting of

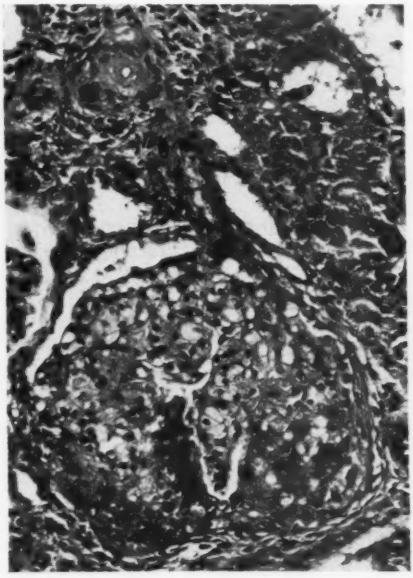

FIG. 5. Kidney. Adrenx. rat, MAD 20 mg+ACE 1 cc. The glomerulus is enlarged and partly necrotic and hemorrhagic. Thickening of afferent arteriole (top left). BP 175 mm. Hg. H and E ×360.

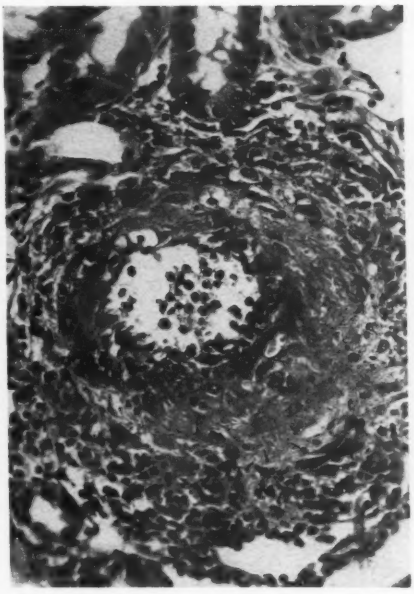

FIG. 6. Kidney. Adrenx. rat, MAD 20 mg.+ACE 1 cc. "Polyarteritis nodosa" involves a small renal artery. BP 162 mm. Hg. H and E ×435.

an increase in intercapillary material with adhesion of tuft to capsule (Fig. 7). Although there was a small average rise in blood pressure in the group, this was not associated with hypertensive vascular lesions in any kidney.

Adrenals. Relative to body weight MAD caused an increase in the weight of the adrenal glands (Table 2). Differences in the distribution of lipid, cholesterol, birefringent crystals and alkaline phosphatase were present on comparison of MAD and control glands. The adrenals from the control sensitized rats showed narrowing and marked lipid depletion of zona glomerulosa. The cells of this zone were small and possessed a compact cytoplasm. Lipid in moderately coarse globules filled the cells of outer zona fasciculata and the reaction became less intense in the inner cortical zone (Fig. 8). The distribution of cholesterol and birefringence followed a similar pattern. Alkaline phosphatase, as demonstrated by our method, was present in the cells and sinusoids of zona reticularis; in the other cortical zones activity was related primarily to the sinusoids with only a slight reaction in the nuclei of the parenchymal cells (Fig. 10).

Following MAD treatment the appearances in zona glomerulosa were similar to those observed in the control glands. In zona fasciculata and reticularis an unusual form of lipid depletion occurred. Cortical cells containing large coarse lipid globules were aggregated in groups while the sur-

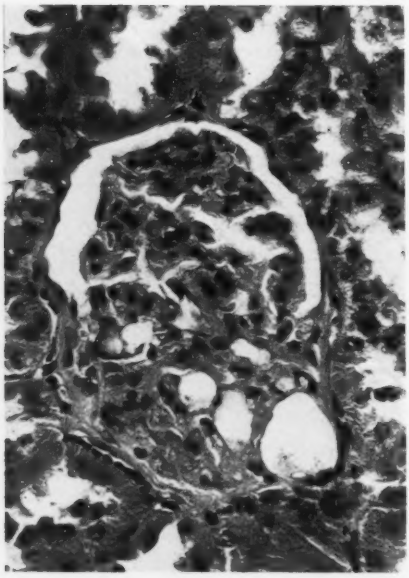

FIG. 7. Kidney. Growth hormone 2 mg. Partial glomerular hyalinization and adhesion of tuft to capsule. BP 151 mm. Hg. H and E ×690.

rounding cells contained fine lipid deposits or were depleted completely. Characteristically, the most uniform depletion occurred in that part of zona fasciculata immediately deep to glomerulosa (Fig. 9). This change was most pronounced with 20 mg. MAD, and in these circumstances the cells were vacuolated although severely depleted of lipid. Reactions for cholesterol and birefringence were related to lipid distribution and were reduced or absent in the lipid depleted areas. Alkaline phosphatase activity, as demonstrated histochemically, was markedly enhanced in MAD glands. The reaction occurred in the inner zone of fasciculata and in zona reticularis. Activity was often confined to focal groups of cells at the 10 mg. dose level (Fig. 11). With 20 mg. MAD the increased reaction was more uniform in distribution, apart from the subglomerular zone of vacuolated cells which showed reduced enzyme localization in comparison with the inner cortex (Fig. 12). A further point of difference between MAD and control glands was the presence in the former of "colloid granules" demonstrable by the PAS stain as described by Skelton (1) and Salgado and Selye (2). The granules were not numerous but were present in all MAD glands. They were localized most commonly to the vacuolated subglomerular zone of fasciculata.

GH caused an absolute increase in adrenal weight (Table 3). Zona glomerulosa showed a similar narrowing and lipid depletion in both test and control rats. There was an increase in width of zona fasciculata and reticularis following GH treatment; the cells were filled with fine lipid droplets and the reactions for cholesterol and birefringence appeared slightly in-

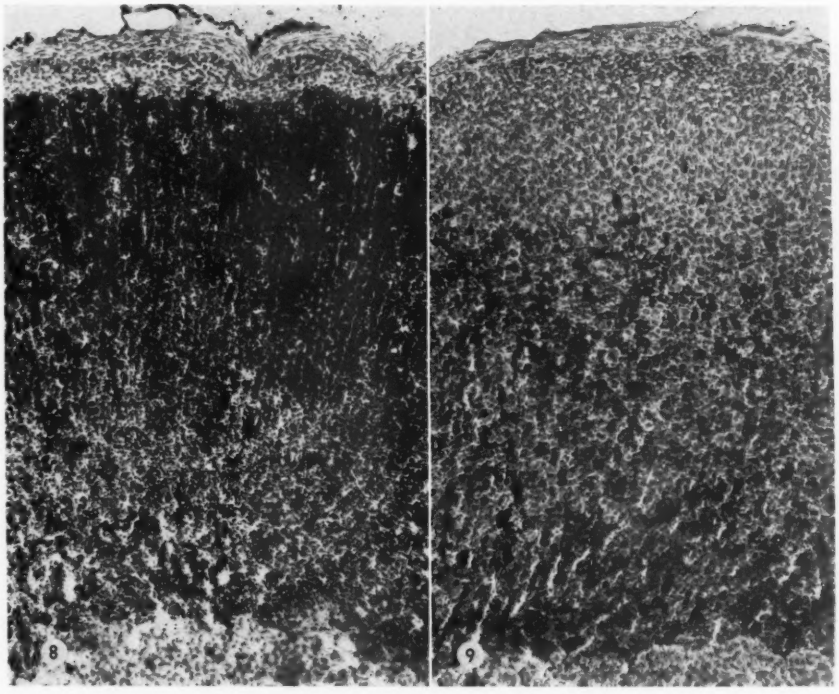

FIG. 8. Adrenal. Control rat. Zona glomerulosa is depleted of lipid. Distribution in fasciculata and reticularis is normal. Oil Red O and H ×157.

FIG. 9. Adrenal. Intact rat, MAD 10 mg. Zona glomerulosa and outer fasciculata are depleted of lipid. Patchy distribution in inner cortex (cf. Fig. 8). Oil Red O and H ×145.

creased. No difference in the distribution of alkaline phosphatase was noted.

Other organs. Examples of hypertensive vascular damage were seen in the *spleen* following MAD treatment in both intact and adrenalectomized rats treated with ACE. Similar lesions were seen also in the *pancreas* of both groups. Salgado and Selye (2) have described a focal "signet-ring" cell change in the pancreatic acinar tissue as a result of MAD administration in both intact and adrenalectomized rats. This has been confirmed in our experiments but a similar appearance was also observed in a control sensitized rat. The *stomach* showed submucosal edema and vascular damage in those rats which were hypertensive; mucosal ulcers were absent.

DISCUSSION

The data of these experiments show that MAD can cause hypertension with renal and cardiovascular pathology in the adrenalectomized-sensitized rat treated with a uniform intake of ACE. The damage occurred

FIG. 10. Adrenal. Control rat. Distribution of alkaline phosphatase. Gomori ×120.

FIG. 11. Adrenal. Intact rat, MAD 10 mg. Focal increase in sites of alkaline phosphatase activity in zona fasciculata and reticularis. Gomori ×135.

FIG. 12. Adrenal. Intact rat, MAD 20 mg. Marked increase in alkaline phosphatase activity apart from zona glomerulosa and subglomerular zone of fasciculata (cf. Figs. 10, 11 and 12). Gomori ×120.

without an increase in food-intake and salt load which is a critical variable in the response of the sensitized rat to cold (5). Although these responses to MAD can occur in the absence of the adrenal glands it is assumed that the presence of adrenal cortical hormones is necessary to support the response since Salgado and Selye (2) have reported that MAD fails to cause such changes in the untreated adrenalectomized-sensitized rat. When the adrenalectomized rats received 2 cc. of ACE per rat per day (Experiment 2) the administration of MAD caused as much damage as occurred in the presence of the adrenals. When 1 cc. of ACE per rat per day was used as substitution therapy (Experiment 3) the administration of MAD caused less damage than occurred in the presence of the adrenals. We assume that the differences noted in Experiment 3 are real, but since the groups are small this is not certain.

Three possible explanations for these results are apparent: (a) the adrenal cortical hormones play a permissive (6) role in the response to MAD; (b) MAD either retards the catabolism of the cortical steroids, thereby causing a state of hypercorticalism, or it favors the formation of cortical steroid metabolites which are pathogenic; (c) MAD may sensitize (7) the tissues to the pathogenic actions of cortical steroids so that a small amount now behaves like a much larger amount. It could be postulated that the alteration in histological and histochemical pattern of the MAD adrenal may be consistent with the production of an abnormal steroid. This does not seem tenable in view of our finding that MAD produced a similar syndrome of pathology in the adrenalectomized rat treated with ACE. Although the authors have some subjective preference for the first hypothesis, none meets any of the important requirements for proof of a proposition.

The unilaterally nephrectomized rat on a high salt load is very sensitive to the level of ACE administered. The small doses used in these experiments elevated blood pressure above that of the control animals and it was previously shown (5, 8) that doses of 4 and 8 cc. per rat per day cause renal and cardiovascular damage. Doses of 1 and 2 cc. of ACE daily are smaller than we have considered to be required for a normalizing dose in adrenalectomized adult rats (9). In these experiments the adrenalectomized rats treated with ACE showed an increase in the weight of thymus which is suggestive of some degree of insufficiency of cortisone-like steroids which affect organic metabolism. Why does the adrenalectomized-sensitized rat, given 2 cc. of ACE daily, appear to have too much cortical hormone in respect to the level of blood-pressure, and too little as indicated by the size of thymus? We have suggested (5) that in the presence of a high salt load the adrenal cortices can suppress their secretion of sodium-retaining steroids without suppression of the release of cortisone-like steroids. The animal on a fixed intake of ACE is unable to regulate its balance of steroids toward the maintenance of homeostasis. This suggestion is

consistent with generally accepted information and concepts (10, 11) relating to the secretion of sodium-retaining steroids by the adrenal cortices.

The preliminary experiment with growth hormone was done on the assumption that we would readily confirm the report of Selye (12) that GH will cause hypertension and nephrosclerosis in the sensitized rat and that we could then extend these studies to an appraisal of the role of the adrenal glands in mediating the damaging effects of GH. Although the growth response of the rats was marked, and although they developed a marked diabetes insipidus and some adrenal cortical enlargement, the elevation in blood pressure was small and the increased damage very mild indeed. In the experiments of Selye (12) the rats drank 1% saline, but there was no added salt in the diet. Rats given GH developed a diabetes insipidus and drank almost four times as much saline as did the controls. In our experience such an increase in salt load can cause elevation of blood pressure with increased renal and cardiovascular damage without the administration of exogenous hormones. In the present experiments the salt was contained in the diet and the rats drank water *ad libitum*. There was a significant increase in the amount of food eaten *ad libitum* which may have contributed to the very small increase in blood pressure and in pathology. On the basis of this experience and these considerations we were discouraged from extending this study of the pathogenic effects of GH in relationship to adrenal cortical function.

Acknowledgment

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THE EFFECT OF CORTISONE IN EXPERIMENTAL HYPERVITAMINOSIS D[1]

WILLIAM C. THOMAS, JR.[2] AND H. GEMMELL MORGAN[3]

Department of Medicine, The Johns Hopkins Medical School and Hospital, Baltimore, Maryland

ABSTRACT

Hypercalcemia is the recognized complication of overdosage with vitamin D and cortisone is sometimes effective in reducing the concentration of serum calcium in this as well as other hypercalcemic states. The present experiment was designed to study the effect of cortisone in experimental hypervitaminosis D.

Rats given 50,000 U. of vitamin D_2 per kilo daily for 7 to 14 days developed hypercalcemia and metaphysial sclerosis. Larger doses (100,000 to 400,000 U.) promoted the formation of excess osteoid tissue and there was an accompanying increase in histological evidence of active bone resorption. In bones of rats given cortisone (50 mg./kg.) the metaphyses were dense and cartilage cell proliferation was reduced. However, concomitant administration of cortisone with vitamin D *did not lessen the vitamin D induced hypercalcemia*, and bones from these rats showed combined effects of both compounds. These results suggest that in rats cortisone and vitamin D act independently rather than competitively on bone.

THE administration of cortisone, or related steroids, has been found effective in reducing the hypercalcemia encountered in sarcoidosis, idiopathic hypercalcemia of childhood, certain neoplastic diseases, the milk-alkali syndrome and in hypervitaminosis D (1, 2, 3). When hypercalcemia exists in association with the foregoing disorders the clinical manifestations are often quite similar and may mimic the syndrome occurring with hypervitaminosis D. Indeed, in sarcoidosis and idiopathic hypercalcemia of childhood an increased sensitivity to vitamin D has been postulated as an explanation for the hypercalcemia (4, 5). Although cortisone administration does not invariably result in amelioration of an existing hypercalcemia (3), it is so often beneficial that it seemed pertinent to study the effect of cortisone in experimental hypervitaminosis D.

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[1] These studies were supported in part by a contract between the Atomic Energy Commission and The Johns Hopkins University.

[2] The work described herein was performed while a Postdoctoral Fellow of the Arthritis and Rheumatism Foundation. Present address: College of Medicine, University of Florida, Gainesville, Florida.

[3] Research Assistant, U.S. Atomic Energy Commission. Present address: Department of Pathology, Royal Infirmary, Dundee, Scotland.

PROCEDURE

Weanling, male, Wistar rats[4] were maintained on Purina chow and water *ad libitum*. When weighing 50 to 65 gm. they were put into groups of 3 to 4 animals per cage and vitamin D and/or cortisone was administered to each rat according to the following schedule: *vitamin* D_2[5] (orally)—400,000, 100,000, 50,000 or 20,000 U./kg. initial body weight daily for 7 to 14 days; *cortisone* (subcutaneously) —50 mg./kg. daily for 7 to 14 days (6), and a few groups were given only 20 mg./kg. daily; *simultaneous cortisone and vitamin D*—the same dosage schedules were used as when either compound was administered singly.

At the time of sacrifice the rats were weighed, etherized and bled by aortic puncture. Serum from rats of each group was pooled for determination of calcium (7), inorganic phosphorus (8), alkaline phosphatase (9) and non-protein nitrogen (10). The proximal tibiae were fixed in neutral formalin for subsequent preparation of undecalcified histological sections (11).

RESULTS

Vitamin D Administration. The results obtained with rats given vitamin D are recorded in Table 1. Rats receiving the largest dose, 400,000 U./kg./

TABLE 1. EFFECT OF VITAMIN D ADMINISTRATION TO RATS

Vitamin D, U./kg./day	Duration of treatment (days)	No. of rats tested	Range of concentrations in pooled sera				Effect on bones		
			Ca, mg.%	P, mg.%	Alk. phosphatase, B.U.	NPN	Excess osteoid	Evidence of active bone destruction	Sclerosis of metaphysis
400,000	12–14	18	15.0–16.5	4.5– 7.4	63	34–43	profuse	marked	yes
400,000	8	10	12.6–15.9	7.1– 7.7	—	42–48	profuse	marked	yes
100,000	14	12	12.1–12.4	8.0–10.4	23–31	33–41	occasional, slight	moderate	yes
100,000	7– 8	14	12.0–13.3	7.0–10.3	26–40	37–38	occasional, slight	moderate	yes
50,000	8	12	11.4–12.0	9.9–11.9	—	38	no	slight	yes
20,000	8	8	10.9–11.5	11.7–12.0	—	41–43	no	no	yes

Listing of only one value indicates data from a single group of rats.

day, usually failed to gain weight, all were hypercalcemic, and several groups were slightly azotemic. The alkaline phosphatase was not low in contrast to values reported for rats treated with larger doses of vitamin D (12).[6] Examination of the tibiae showed some decrease in width of the zone of proliferative cartilage. Due to persistence of calcified cartilage matrix surrounded by new bone and excessive osteoid tissue the metaphyses were much wider than normal. However, in the rats given the vitamin for 14 days active bone resorption had rendered the mid-metaphysial area almost devoid of bone. This resorptive process in the metaphysis was characterized by the presence of partially destroyed trabeculae, large vascular spaces and numerous osteoclasts. Throughout the tibiae there was abun-

[4] Obtained from Research Supply Co., Philadelphia, Pennsylvania.

[5] Obtained from C. F. Kirk Co., New York, New York.

[6] Apparently serum alkaline phosphatase of rats is largely of intestinal origin and may be altered by a variety of means (13). Severe vitamin D intoxication has been shown to reduce the phosphatase activity in rat serum (12, 14), but as starvation also lowers the serum phosphatase in this species, a specific effect cannot yet be attributed to excess vitamin D per se.

dant osteoid tissue. Both narrowing of the proliferative cartilage zone and excessive bone resorption were less marked in rats given vitamin D for only 7 days (Fig. 1C and D).

Groups of rats given 100,000 U./kg./day gained weight and were hypercalcemic, but the changes in the bones were much less striking than in those receiving larger doses of vitamin D. The metaphyses were wide and dense, but the zones of proliferative cartilage were not narrowed, and only scattered foci of increased bone resorption were evident (Fig. 1B). In two-thirds of the rats, whether treated for 7 or 14 days, there was no abnormal amount of osteoid tissue, and bones of the remaining rats showed only occasional areas of excessive osteoid tissue. Similar but less extensive changes were present in the tibiae of rats given 50,000 U./kg., a dose which induced only slight hypercalcemia. Rats receiving lesser amounts of the vitamin (20,000 U./kg.) remained normocalcemic and had only slightly increased width and density of the metaphyses.

TABLE 2. EFFECT OF CORTISONE ADMINISTRATION TO RATS

Cortisone, mg./kg./day	Duration of treatment (days)	No. of rats tested	Range of concentrations in pooled sera				Effect on bones	
			Ca, mg.%	P, mg.%	Alk. phosphatase, B.U.	NPN, mg.%	Narrow cart. plate	Metaphysial sclerosis
50	13–14	12	10.2–10.4	8.1–8.4	—	34	yes	marked
50	8	8	9.0–11.0	7.3	45	32	yes	marked
20	14	3	11.0	9.5	—	48	slight	moderate
20	7	5	10.7	8.2	66	46	slight	moderate

Listing of only one value indicates data from a single group of rats.

Cortisone Administration. The data obtained on rats given cortisone are recorded in Table 2. Rats receiving daily doses of 50 mg./kg. of initial weight for 8 to 14 days usually failed to gain, whereas those given 20 mg./kg. had a slight weight increase. The concentration of calcium in pooled sera from each group of these animals was normal. The histopathology of bones from cortisone treated rats has been well described by Follis (6). Similar changes noted in this experiment consisted of a narrowed zone of proliferative cartilage, a dense metaphysis with persistence of calcified cartilage matrix enveloped by new bone, and little or no evidence of remodeling of trabeculae in the proximal metaphysis (Fig. 2). A dense and widened metaphysis developed also in rats receiving 20 mg./kg. daily, but there was less inhibition of cartilage cell proliferation than when larger amounts were used.

Simultaneous Cortisone and Vitamin D Administration. The observations made on rats given both cortisone and vitamin D are recorded in Table 3. The animals treated with this combination of drugs lost weight when large doses were used and even with smaller doses the weight gain was less than with either compound alone. Concomitant cortisone administration *did not lessen the hypercalcemia* induced by vitamin D. This was true despite use of the maximal tolerated dose of cortisone (50 mg./kg.) in con-

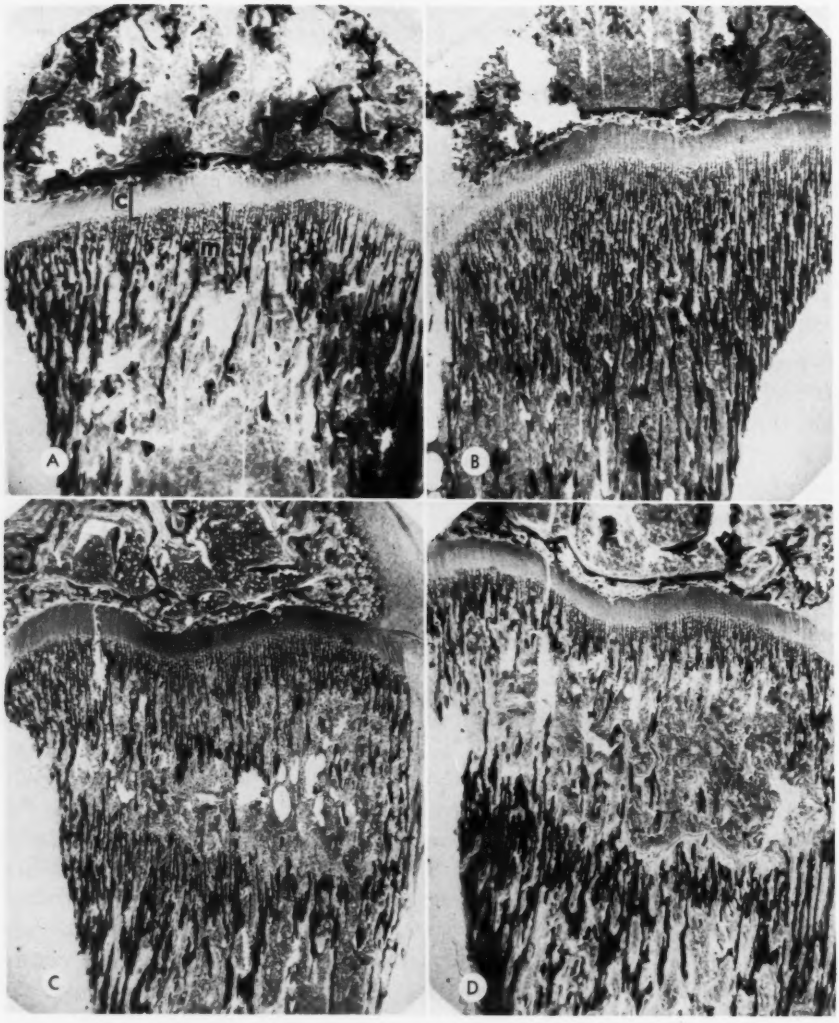

FIG. 1. Undecalcified sections of proximal tibiae stained with $AgNO_3$, hematoxylin and eosin (×14).

A. Normal control. Note width of proliferative cartilage zone (c) and metaphysis (m).

B. Vitamin D, 100,000 U./kg./day for 1 wk. Metaphysis wide and dense.

C. Vitamin D, 400,000 U./kg./day for 8 days. Metaphysis wide, but much resorption of bone has occurred in mid-metaphysis. Osteoid tissue present along some trabeculae.

D. Vitamin D, 400,000 U./kg./day for 2 wk. Mid-metaphysis almost completely devoid of trabeculae. Osteoid tissue abundant.

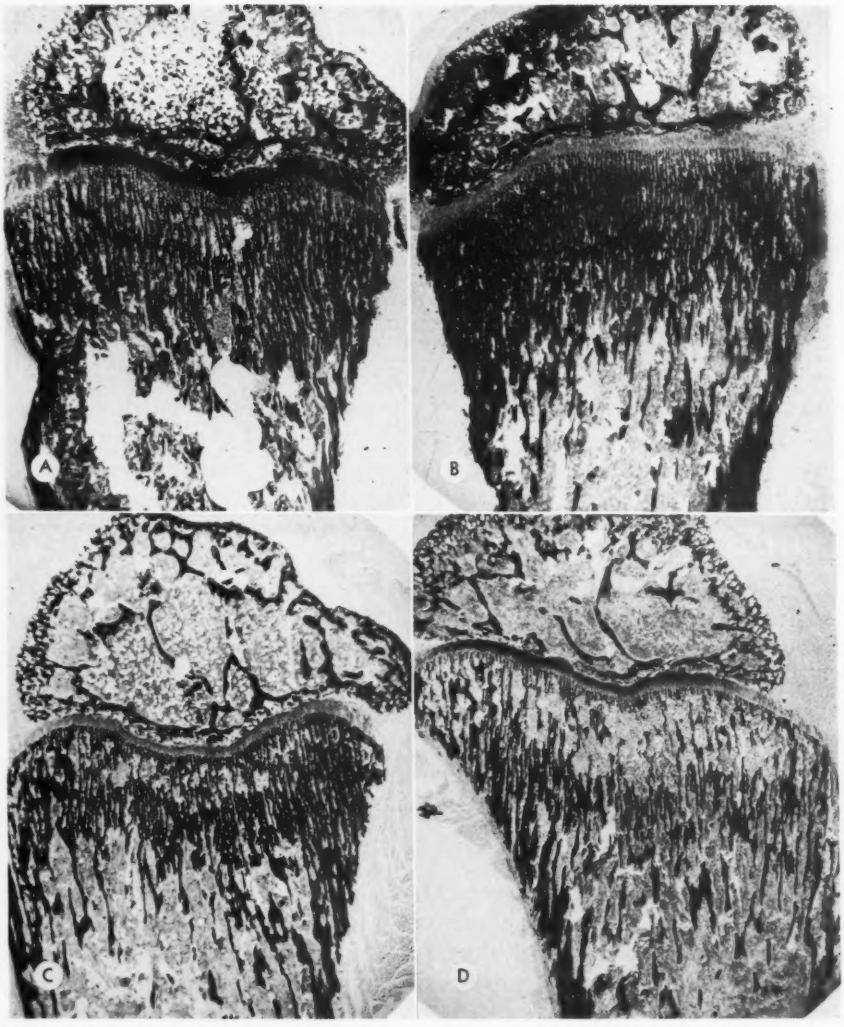

FIG. 2. Undecalcified sections of bone stained as in Figure 1 (×14).

A. Cortisone 50 mg./kg./day for 8 days. Zone of proliferative cartilage narrow. Metaphysis wide and dense.

B. Cortisone as in 2A, but for 14 days. Extremely dense metaphysis. Width of epiphysial cartilage slightly less than in 2A.

C. Cortisone 50 mg./kg. and vitamin D 100,000 U./kg. daily for 8 days. Areas of active bone resorption in proximal portion of dense metaphysis. Compare with Figure 1B and 2A.

D. Cortisone 50 mg./kg. and vitamin D 400,000 U./kg. daily for 8 days. Same as in 2C except for more evidence of bone resorption in proximal metaphysis and osteoid tissue is present about many trabeculae. Compare with 1C and 2A.

TABLE 3. EFFECT OF SIMULTANEOUS CORTISONE AND VITAMIN D ADMINISTRATION TO RATS

Vitamin D, U./kg./day	Cortisone, mg./kg./day	Duration of treatment (days)	No. of rats tested	Range of concentrations in pooled sera			
				Ca, mg.%	P, mg.%	Alk. phosphatase, B.U.	NPN mg.%
400,000	50	12–14	12	12.4–15.5	7.2– 7.7	—	34–54
400,000	50	8	10	14.2	8.0	45	84
100,000	50	8	12	12.9–13.3	9.6–10.3	45–71	34–44
100,000	20	14	11	12.6–12.8	8.4– 9.9	29–30	35
100,000	20	7	10	13.2–13.6	7.2– 8.7	35–36	46–52
50,000	50	18	16	11.3–11.9	10.8–11.2	—	35
20,000	50	12	12	10.5–10.8	9.0–11.7	—	44–47

Listing of only one value indicates data from a single group of rats.

junction with the minimal amount of vitamin D necessary to induce hypercalcemia (50,000 U./kg. for 8 days). The structural derangement in the tibiae of these rats was complex and consisted of a combination of those changes observed after either cortisone or vitamin D administration (Fig. 2C and D). Although excess osteoid tissue was present in rats given cortisone plus 400,000 U. of vitamin D/kg. and in a few given 100,000 U./kg., it was less profuse than with vitamin D alone at similar doses. In addition, cortisone effect was evidenced by the markedly narrowed zones of proliferative cartilage, increased sclerosis of metaphyses, and less bone resorption than with vitamin D alone. The osteoblasts surrounding trabeculae in vitamin D treated animals were large with vesicular nuclei, but the addition of cortisone resulted in a diminution in number and size of these cells. At all dosage schedules used in this experiment some histological effect of both compounds could be seen in the bones.

DISCUSSION

Experimental hypervitaminosis D is characterized by hypercalcemia and a variety of changes in the bones of animals. The particular effect achieved is influenced by diet, duration of treatment and amount of vitamin D given (15). A moderate excess of vitamin D to young animals results in sclerosis of the metaphyses (16, 17, 18), whereas larger doses or prolonged treatment causes histologically evident bone destruction. Although noted previously (16) (18), it was Ham and Lewis (19) who emphasized the production of excessive osteoid tissue in animals given large amounts of vitamin D.

In the present experiment all of the above mentioned changes in sera and bones were noted. Of interest was the difference in dosage of vitamin D required to produce the particular manifestations of hypervitaminosis D. One thousand units (20,000 U./kg.) of vitamin D daily for 1 to 2 weeks induced metaphysial sclerosis. In the same time period, administration of approximately 2,500 units (50,000 U./kg.) daily resulted in hypercalcemia and histological evidence of increased bone resorption, whereas 5,000 to

20,000 units (100,000 to 400,000 U./kg.) daily promoted excess osteoid tissue formation in addition to an increase in the effects noted after smaller doses.

Clinically, hypercalcemia is the recognized complication of overdosage with vitamin D. Although occurring infrequently, radiologically demonstrable changes in bone have been noted in hypervitaminosis D of children (20, 21). The metaphysial sclerosis with mid-metaphysial rarefaction reported by Ross (21) appears quite analogous to the experimentally induced osseous changes following vitamin D administration (16) (Fig. 1). To our knowledge hypervitaminosis D rickets has not been encountered clinically, and the results obtained in this experiment afford a possible explanation. To promote excess osteoid tissue formation required 2 to 8 times the dosage of vitamin D necessary to induce hypercalcemia. Thus, if a similar dose response ratio is applicable to humans, extremely large amounts of the vitamin would be necessary to cause hypervitaminosis D rickets.

In this study hypercalcemia induced by vitamin D was not modified by concomitant cortisone administration. These results contrast with clinical experiences in which cortisone has proven effective in reducing vitamin D induced hypercalcemia (3). It is possible that had the vitamin D been given prior to rather than concomitantly with cortisone different effects may have occurred. In view of the persistence of vitamin D in the serum long after cessation of therapy (22) such a sequence seems of doubtful importance. However, in two instances of vitamin D induced hypercalcemia in which the vitamin was continued during cortisone therapy, the concentration of serum calcium was unaffected in one patient and reduced in the other (23).

The mode of action whereby large doses of cortisone reduce the calcium concentration in some patients with hypercalcemia is unknown. One suggestion is that cortisone may inhibit the effect of the hypercalcemia-inducing substance through competitive action on target enzyme systems of bone (23). Recently, evidence in support of such an action has become available. Neuman and Neuman (24) have compiled data which suggest that vitamin D enhances formation of oxaloacetate and acetyl coenzyme A from pyruvate, whereas Henneman and Bunker (25) have indicated that hydrocortisone inhibits this reaction. Nevertheless, failure of cortisone to lessen vitamin D induced hypercalcemia in rats and the presence of distinct osseous effects of both compounds suggest that these agents may act independently on bone in this species.

To our knowledge inhibition of bone destruction in man by cortisone has not been demonstrated histologically except possibly in Paget's disease (26). However, the effectiveness of cortisone in lessening hypercalcemia of varied etiology in patients on calcium free diets (3) constitutes indirect evidence that on these occasions, at least, cortisone does inhibit bone resorption. In rats the short term administration of cortisone inhibits bone resorption with consequent development of dense metaphyses. An

explanation for this effect, so different from that usually observed in man, is not apparent, but two factors warrant consideration. [1] The dosage of cortisone used, 20 to 50 mg./kg., is apparently in excess of that amount of corticosteroids maximally produced by the rat (27) and smaller doses may have induced changes different from those observed in this experiment. [2] Corticosterone rather than cortisone is the major corticoid hormone of rat adrenal glands (27), and the action of this hormone on rat bones and on vitamin D induced hypercalcemia may not be identical to that of cortisone.

Acknowledgment

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IN VITRO EFFECTS OF ADRENAL CORTICAL STEROIDS ON TRANSAMINATION BY CELL SUSPENSIONS OF RAT THYMUS AND LYMPHOSARCOMA[1]

MELVIN BLECHER AND ABRAHAM WHITE

Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, N. Y.

ABSTRACT

Aspartate-glutamate transaminase activities have been determined in cell suspensions of thymic lymphocytes from normal and adrenalectomized pre- and post-pubertal rats and of the transplantable Murphy-Sturm lymphosarcoma. This activity is lower in thymic lymphocytes obtained from younger than from older normal animals. The *in vitro* addition of hydrocortisone or of cortisone to such suspensions decreased, or increased, by approximately 40% the transaminase activity of thymic lymphocytes from pre- and post-pubertal rats, respectively. Deoxycorticosterone, testosterone and 17α-ethyl-19-nortestosterone were without influence on transaminase activity in lymphocyte suspensions. Although adrenalectomy may elevate baseline transaminase or depress transaminase activity, in thymic lymphocytes from younger and older rats, respectively, the *in vitro* addition of hydrocortisone to such suspensions returns transaminase activities to levels seen with normal animals. Lymphocytes obtained from the lymphosarcoma show greatly elevated transaminase activities, and are unaffected by the *in vitro* addition of hydrocortisone. Thymic lymphocytes from the tumor-bearing host did respond in typical fashion to the presence of steroid. Castration results in markedly decreased transaminase activity in thymic lymphocytes from rats of both age groups. However, transaminase activity in lymphocytes from rats castrated postpubertal responds in typical fashion to the presence of hydrocortisone. In contrast, rats castrated at a pre-pubertal age continue, at an older age, to have lymphocytes which show the response to hydrocortisone typical of lymphocytes from younger animals.

Preincubation of lymphocytes with hydrocortisone prior to homogenization, yields cell-free fractions which showed a marked increase in transaminase activity.

A VARIETY of experimental procedures has been employed to induce lymphoid tissue involution in animals, e.g., mice and rats. Pyridoxine deficiency, produced by feeding a diet deficient in the vitamin and injecting the metabolic antagonist, deoxypyridoxine, caused a marked atrophy of thymic tissue in mice (1). The apparently specific effect of even relatively

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mild pyridoxine deficiency in producing lymphoid tissue involution suggests that pyridoxine may play an essential role in the metabolism of lymphoid tissue. Pyridoxal phosphate is the coenzyme component of the transaminases and of other enzyme systems concerned with aspects of amino acid and protein metabolism. In view of the interrelations between certain of the adrenal cortical steroids and the contributions of lymphoid tissue to protein metabolism (2, 3), a study has been conducted of the transaminase activity of lymphocyte suspensions obtained from the thymus of normal and adrenalectomized rats, and from a transplantable lymphosarcoma of the rat. In addition, the effects of certain steroids, added *in vitro*, on the transaminase activity of lymphocyte suspensions have been examined.

EXPERIMENTAL

Male rats of the Sprague-Dawley strain[2] were employed in all experiments. Both prepubertal animals, less than 6 weeks of age and weighing 100 to 140 gm., and postpubertal rats, 10 to 14 weeks of age and weighing 240 to 280 gm., were used. Adrenalectomized rats were studied 14 days following bilateral adrenalectomy. All animals were fed Rockland pellets and received water *ad libitum*, except the adrenalectomized animals which were given 1% NaCl solution as drinking fluid. In experiments in which testosterone was administered to weanling rats, each animal received daily, subcutaneous injections of 0.5 mg. testosterone propionate[3] in 0.2 ml. sesame oil for 2 weeks. Gonadectomy was performed in weanling rats and the animals sacrificed for study 7 weeks later; the same operative procedure was carried out in 10 week old rats and the thymus tissue obtained at sacrifice 14 days after castration. The transplantable Murphy-Sturm lymphosarcoma[4] was allowed to develop subcutaneously for 14 days after the initial transplant prior to sacrifice of the host.

Washed cell suspensions from thymic tissue obtained from the rats were prepared essentially as described by Farber, Kit and Greenberg (4) in their modified Krebs-Ringer phosphate buffer, pH 7.4. An initial centrifugation[5] at $150 \times g$ for 1 minute removed tissue debris. Cell suspensions from tumor tissue were obtained by mincing the tissue with fine scissors and decanting the cells with use of buffer; cells were then isolated and suspensions prepared as for thymic lymphocytes. All counts were made on diluted aliquots of the suspensions in a hemocytometer, using 1:4000 Safranin O in Tyrodes' solution (5). This also provided an index of viable cells. Cell suspensions contained approximately 1.5×10^6 cells per ml. Dry weight determinations showed that 10^6 thymus or lymphosarcoma cells weigh approximately 50 and 150 μgm., respectively (6). Identification of the cells employed as lymphocytes was confirmed by staining air-dried imprints or frozen sections[6] with hematoxalum or Wright's-Giemsa stains.

[2] Purchased from the Holtzman Co., Madison, Wisc.

[3] The steroids used in this study were generously provided from the following sources: hydrocortisone, Merck, Sharpe and Dohme Co., Rahway, N. J.; deoxycorticosterone, testosterone, and testosterone proprionate in oil (Oreton propionate), Schering Corp., Bloomfield, N.J.; 17α-ethyl-19-nortestosterone (Nilevar), G.D. Searle and Co., Chicago, Ill.

[4] Grateful acknowledgement is made to Dr. K. Sugiura, Sloan-Kettering Institute, New York, for providing the initial lymphosarcoma for transplantation in these studies.

[5] All centrifugations described in this paper were conducted at 0° C in a refrigerated International Centrifuge, model PR-2.

[6] We are indebted to Dr. A. Novikoff of the Department of Pathology for preparing the frozen sections.

Broken cell preparations of thymic tissue from post-pubertal rats were prepared by grinding 10% suspensions of thymus lymphocytes in phosphate buffer in a Potter-Elvehjem-type, all-glass homogenizer for three 1-minute periods at 0° C. Cell-free extracts of these preparations were prepared by centrifugation at 700×g for 10 minutes. A microsomal-cytoplasmic fraction was obtained by centrifuging the supernatant fluids at 5,000×g (Multi-speed attachment) for 10 minutes followed by recentrifugation of the washed mitochondrial pellet at 24,000×g for 10 minutes. Protein determinations were performed both by a semi-micro Kjeldahl procedure and by a microbiuret method (7).

Incubations were done in Warburg vessels. Appropriate quantities of the steroid studied were first added to each vessel in ethanolic solution and solvent removed with a current of warm air; final concentrations varied from 1 to $8\times10^{-4}M$. In manometric experiments the main compartment contained 0.5 ml. of cell suspension, 100 μmols. buffered sodium L-aspartate,[7] 0.12 μmols. pyridoxal-5-phosphate[8] and sufficient additional buffer to make a final volume of 2.5 ml. One side-arm contained 50μmols. α-ketoglutarate[7] and the second side-arm 0.5 ml. of aniline citrate-citric acid (8). After a 15 minute preincubation period at 37° C, the reaction was started by tipping in the keto acid. Incubations were conducted in pre-purified N_2 at 37° C with shaking and were terminated by tipping in the aniline citrate. Release of CO_2 from oxaloacetate formed by transamination was measured manometrically.

The manometric data obtained were checked by colorimetric determinations (9) of the quantity of oxaloacetate formed and α-ketoglutarate remaining after incubation. In such balance studies the concentration of substrates was reduced by four-fifths and the period of incubation increased from 30 to 90 minutes. Results obtained by the two methods of transaminase assay agreed within 5%.

RESULTS

The viability of the lymphocyte suspensions during the maximum 90 minute period of incubation, with or without steroid addition, was suggested from counts of stained cells. More significant in this regard are data from unpublished studies in which oxygen consumption of similarly prepared lymphocyte suspensions has been observed to continue briskly for as long as a 7 hour period of study.

Preincubation of lymphocyte suspensions with pyridoxal-5-phosphate alone for periods longer than the 15 minute preincubation period used in the presence of substrate (see above), did not increase transaminase activity above that seen without use of extra coenzyme.

In Table 1, the mean basal values for transaminase activity of various lymphocyte suspensions are given in the column of values labeled as controls, transaminase activity. It will be seen that lymphocytes from post-pubertal rats appear to have a higher transaminase activity than that of cells from younger animals, whereas following adrenalectomy, the higher transaminase activity is observed in thymic lymphocytes from the younger age group. The high level of transaminase activity in the lymphosarcoma

[7] California Foundation for Biochemical Research, Los Angeles, California.

[8] Two preparations of pyridoxal-5-phosphate were employed with similar results being obtained. One preparation was generously supplied by Merck, Sharpe and Dohme, Rahway, N. J.; the other was obtained from the California Foundation for Biochemical Research, Los Angeles, California.

TABLE 1. EFFECT OF HYDROCORTISONE *in vitro* ON ASPARTATE-GLUTAMATE TRANSAMINASE ACTIVITY OF SUSPENSIONS OF RAT LYMPHOCYTES*

Type of rat providing lymphocytes used	Controls, transaminase activity†	Mean % change of transaminase activity from control levels produced by addition of hydrocortisone				
		Concentration of hydrocortisone, M $\times 10^{-4}$				
		1	2	3	4	5
1. Normal, 3–6 weeks of age	396 ± 56‡(10)§	−16 (2) (14–18)‖	−27 (8) (20–31)	−37 (4) (32–40)	−39 (10) (30–50)	−44 (2) (35–48)
2. Normal, 10–14 weeks of age	620 ± 42‡(15)	+13 (4) (10–20)	+28 (9) (12–40)	+31 (3) (16–52)	+37 (9) (21–63)	+44 (2) (38–49)
3. Adrenalectomized, 3–6 weeks of age	740	−10	−23	−32	−34	
4. Adrenalectomized, 10–14 weeks of age	244	+47	+72	+82	+110	
5. Lymphosarcoma-bearing, 10–14 weeks of age						
Thymus cells	1,062 (2) (544–1570)		+22 (2) (18–25)		+44 (2) (43–45)	
Lymphosarcoma cells	7,475 (2) (4,050–10,900)		0 (2) (−13, +12)		−10 (2) (−2, −18)	
6. Testosterone treated, 3–6 weeks of age	745	− 3	− 9	−35	−45	−39
7. Castrated at 10 weeks of age: 12 weeks old	58	+10	+19	+28	+33	+38
8. Castrated at 3 weeks of age: 13 weeks old	118	− 5	−20	−37	−39	−42

* All lymphocyte suspensions used were from thymic tissue, except in the csae of the lymphosarcoma cells.
† Transaminase activity = μmol. oxaloacetate formed/hr./gm. dry wt. cells.
‡ Mean values and standard errors. Value of t for comparison of experiments 1 and 2 = 3.20; $P < 0.005$.
§ Number of individual experiments indicated in parentheses for given steroid concentration.
‖ Range of values. Values in parentheses showing range for a mean with a negative sign are each negative.

cells is noteworthy, as is the apparent effect of the presence of the tumor in elevating transaminase activity in the thymic cells of the host. A similar augmenting effect in the presence of the transplantable lymphosarcoma on the metabolic activity of thymic lymphocytes of a tumor-bearing host has been observed (10) in *in vitro* measurements of protein and nucleic acid formation. Treatment of prepubertal rats with testosterone propionate increased markedly transaminase activity of thymic lymphocytes to levels characteristic of cells from older animals. On the other hand, castration of either prepubertal or postpubertal rats led to a marked decline in transaminase levels in lymphocytes.

In Vitro Effects of Steroids on Aspartate-Glutamate Transaminase Activity of Lymphocytes

Transaminase activities in thymus lymphocytes from normal animals were affected in proportion to the concentration of hydrocortisone used up to a maximum concentration of $5 \times 10^{-4} M$. With higher concentrations of hydrocortisone, no further effect on transaminase activity was observed. However, the direction of this change in transaminase level was dependent on the age of the animals employed. Addition of hydrocortisone to thymic lymphocytes from *prepubertal* animals *inhibited* transaminase activity, whereas the presence of steroid with lymphocytes from *postpubertal* animals *augmented* transaminase activity of these cells.

The data obtained with hydrocortisone addition to lymphocytes from adrenalectomized rats (Table 1, lines 3 and 4) were qualitatively the same as with normal rats. Control levels of transaminase activity were, however, higher in lymphocytes from adrenalectomized, prepubertal rats, and lower

in lymphocytes from adrenalectomized, postpubertal animals, as compared with cells from their unoperated controls of appropriate ages. Nevertheless, the direction and degree of the effect of added hydrocortisone were the same as with lymphocytes from normal rats, i.e., a decrease in transaminase activity when the lymphocytes were derived from thymi of prepubertal, adrenalectomized rats, and an increase in enzyme activity when the thymic cells were taken from postpubertal, adrenalectomized rats.

The hydrocortisone effect on lymphocytes from the thymus of the lymphosarcoma-bearing, postpubertal rat is qualitatively the same as that observed for thymic cells from normal rats (Table 1, lines 2 and 5). However, transaminase activity of the lymphosarcoma cells is unaffected by the presence of hydrocortisone. Data similar to those obtained with hydrocortisone have been obtained with comparable concentrations of cortisone. In contrast, deoxycorticosterone, testosterone and 17α-ethyl-19-nortestosterone, added in concentrations similar to those of hydrocortisone, were found to be totally without influence on the transamination processes in thymic lymphocytes from either young or older animals.

Effect of Castration or of Testosterone Treatment on Aspartate-Glutamate Transaminase Activity of Lymphocytes

Prior castration of either pre- or post-pubertal rats reduced to very low levels the transaminase activity of thymic lymphocytes obtained from these animals (Table 1, lines 7 and 8). Daily injections of testosterone propionate for 2 weeks in prepubertal castrate rats increased the transaminase activity of thymic lymphocytes to levels considerably above those seen with cells from intact control animals of similar age; the values attained were characteristic of lymphocytes from older animals (Table 1, compare line 6 with lines 1 and 2). However, castration of postpubertal animals and testosterone treatment of prepubertal animals did not alter the hydrocortisone effect on the transaminase process in thymic lymphocytes (Table 1, lines 6 and 7). On the other hand, castration of rats at 3 weeks of age prevented the change in the direction of hydrocortisone effects seen normally as rats pass through puberty into the postpubertal state (Table 1, line 8). Thymic lymphocytes from these older animals castrated at 3 weeks of age responded to hydrocortisone addition with a decrease in transaminase activity; the direction of this change is characteristic of lymphocytes of prepubertal rats (Table 1, line 1).

Hydrocortisone Effects on Cell-free Extracts of Thymic Lymphocytes

Hydrocortisone effects on aspartate-glutamate transaminase activity of cell-free, soluble extracts derived from rat thymus lymphocytes are shown in Table 2. Hydrocortisone addition *in vitro* had no consistent influence on the transaminase activity of either total broken-cell preparations or a low-speed supernatant fluid derived from these preparations (Table 2, Exps. I

TABLE 2. ANAEROBIC TRANSAMINATION IN CELL FRACTIONS PREPARED FROM THYMIC LYMPHOCYTES*

Expt.	Preparations†	R.C.F.‡	Transamination§	% increase
I	WH	—	0.79	
	WH + 60μg.F/ml.	—	0.77	0
	HS	700	4.99	
	HS + 60μg.F/ml.	700	5.21	5
II	Su	700	3.56	
	Su + 60μg.F/ml.	700	5.16	45
	Sp	700	8.23	130
III	Su	24,000	3.11	
	Su + 120μg.F/ml.	24,000	3.46	11
	Sp	24,000	6.52	109
IV	Su	24,000	4.03	
	Su + 120μg.F/ml.	24,000	3.88	0
	Sp	24,000	8.67	115

* Thymic tissue of rats 10–14 weeks of age was used for all studies in this table.
† WH, broken-cell preparations of thymus; HS, supernatant fluid from WH; F, hydrocortisone; Su, supernatant from broken-cell preparations of control lymphocytes; Sp, supernatant from broken-cell preparations of lymphocytes preincubated with F.
‡ R.C.F., relative centrifugal force, ×g.
§ Oxalocetate formed in μmols./hr./mg. protein.

and II). However, preincubation of thymus cell suspensions with $2.2 \times 10^{-4}M$ hydrocortisone (for 30 minutes at either 25° or 37° C with shaking) prior to homogenization resulted in supernatant fluids (Table 2, Exps. II, III and IV) in which transaminase activity was more than twice as great as in controls.

DISCUSSION

Interpretation of alterations in levels of transaminase activity *in vivo* is made more difficult by the fact that transaminases catalyze reversibly reactions which can be interpreted as either anabolic or catabolic. The central role of α-ketoglutarate in transamination, as an acceptor *in vivo*, under the catalytic influence of specific transaminases, of amino groups from all amino acids, except threonine and lysine, to form glutamic acid focuses attention on transamination as a key mechanism in the initial catabolism of amino acids. Moreover, the subsequent enzymatic removal of the nitrogen of glutamic acid, catalyzed by L-glutamic acid dehydrogenase, channels the nitrogen of amino acids into irreversible pathways of urea formation and excretion. Viewed in this manner, transamination provides a mechanism for continually removing from the body nitrogen from amino acids derived from dietary or tissue sources in excess of the needs of the organism. On the other hand, transamination of glutamic acid with α-keto acids which can be synthesized by the mammal is a basis for the biosynthesis of a variety of amino acids which are not essential in the diet. From this point of view, transaminases may function to promote anabolic processes.

In general, circumstances which favor growth have been reported to be accompanied by a decrease in tissue transaminase activity. This may be interpreted as directed toward preservation of dietary amino acids for growth purposes. Thus, Cohen and Hekhius (11) observed low transaminase activity in rapidly growing embryonic, malignant or regenerating tissues. Bartlett and Glynn (12), Gaebler (13) and Zuchlewski and Gaebler (14) have reported an abnormally high glutamate-aspartate transaminase in liver and muscle of hypophysectomized rats. These high values were reduced by treatment of the animals with growth hormone. Beaton, Curry and Veen (15) found that in adult rats, single or long term injections of growth hormone reduced liver glutamate-alanine transaminase activity. In contrast, cortisone administration has been reported (15–17) to increase significantly glutamate-aspartate and glutamate-alanine transaminase activity in both normal and regenerating liver of rats, without affecting the rate of hepatic regeneration (17). It was suggested that the hormone may enhance gluconeogenesis by activating the transamination process (17).

The hazards in interpreting increases or decreases in tissue transaminase levels in terms of amino acid anabolism or catabolism are emphasized by the findings of Zuchlewski and Gaebler (14). Despite the drastic reduction of liver glutamate-alanine transaminase activity produced in hypophysectomized rats treated with growth hormone, transfer of isotopic nitrogen from alanine to other amino acids occurred to the same extent in the growth hormone-treated animals as in appropriate controls. Thus, a marked decrease in measurable enzyme activity was not reflected in an interference with the extent of enzyme functioning.

The relating of data from transaminase studies on tissues obtained from animals treated with hormones *in vivo* to findings in the present study with cells treated with hormones *in vitro*, has definite limitations. Nevertheless, except for the high levels of transaminase activity in the lymphosarcoma cells, the data fit a discernible pattern which is in general agreement with previous concepts. The elevated transaminase activity of lymphosarcoma, as compared to normal thymic, lymphocytes, is in contrast with data (11) for other malignant tissue, but is generally in agreement with higher transaminase values found by Kit and Awapara (18) for the Murphy-Sturm lymphosarcoma, and other lymphosarcomas, as compared with normal thymus or spleen. The higher transaminase values found in lymphocytes from postpubertal as compared with prepubertal rats could be a reflection of the continuing growth status of the younger animals, and perhaps a consequence of increased availability of growth hormone. The nature of the effects of hydrocortisone added *in vitro* on transaminase activity of lymphocytes showed a striking dependency on the age of the animals from which the tissue was obtained. Added to lymphocytes from prepubertal animals, hydrocortisone decreased transaminase activity. In contrast, under identical experimental conditions, hydrocortisone augmented transaminase ac-

tivity in lymphocytes obtained from postpubertal animals. In the latter instance, it could be inferred that the hormone was exerting a transaminase influence reflecting its catabolic activity (17, 19), whereas in the case of the lymphocytes from younger animals, the hormone's effect on transaminase activity is a reflection of the anabolic activities of the younger rats. The failure of added hydrocortisone to influence transaminase activity in lymphosarcoma lymphocytes is noteworthy and in contrast with hormonal effects seen with thymic lymphocytes from the tumor-bearing rat.

It may be noted that the direction of the *in vitro* effect of hydrocortisone on transaminase activity of lymphocytes was also dependent on the age of the donor rat when the cells were obtained from adrenalectomized animals. The higher then normal basal transaminase activity of lymphocytes from prepubertal, adrenalectomized rats, and the lower than normal values for lymphocytes from postpubertal, adrenalectomized rats could be a result of the absence of the adrenal steroid influence in the operated animal, i.e., to diminish transaminase activity in lymphocytes from prepubertal rats and to augment this activity in older animals. The levels of transaminase activity of lymphocytes from adrenalectomized rats would thus reflect the absence, prior to sacrifice of the animals, of circulating adrenal cortical steroids.

An influence of testosterone on the transaminase activity of lymphocytes is clearly indicated from the data. Awapara (20) reported that castration in the rat decreases considerably the transaminase activity of the ventral prostate and seminal vesicles. Kochakian and Endahl (21) found an increase in the transaminase activities of the temporal muscles of guinea pigs treated with anabolic steroids, and related this to a generalized increase in muscle weight. In the present study, castration of either younger or older rats produced a subsequent striking decline in transaminase activity of thymic lymphocytes of these animals. Moreover, testosterone treatment of prepubertal animals elevated transaminase activity markedly. However, testosterone treatment of prepubertal rats, or castration of postpubertal animals, did not alter the direction of the added hydrocortisone effect on lymphocyte transaminase activity. On the other hand, lymphocytes obtained from older rats which had been castrated prior to puberty responded to hydrocortisone addition with a decrease in transaminase activity, a response characteristic of the cells of prepubertal animals. The presence of testosterone appears necessary for the augmenting effect of added hydrocortisone on transaminase activity of lymphocytes from older animals.

The effects of hydrocortisone added *in vitro* on lymphocyte transaminase activity were characteristic for this steroid and were in contrast to the complete absence of a discernible effect by deoxycorticosterone, testosterone, and 17α-ethyl-19-nortestosterone. These results, taken together with

studies of the morphology of the cell suspensions at the end of the incubation period and unpublished measurements of oxygen consumption, suggest that the results obtained with hydrocortisone were due to specific actions of this steroid. Moreover, other studies (10) of lymphocyte metabolism have shown that deoxycorticosterone and 17α-ethyl-19-nortestosterone may alter protein and nucleic acid metabolism in lymphocytes. The data support the suggestion that specific steroids may influence selected metabolic phenomena in lymphocytes.

Transamination studies with fractions of broken-cell preparations of lymphocytes were initiated as an effort to examine the mechanism of action of hydrocortisone on transaminase activity of lymphocytes. These experiments were expected to give a beginning insight into whether steroid action at the cell surface and variable permeability of lymphocytes to diffusible ions or substrates might contribute to the data obtained. Added hydrocortisone failed to influence consistently transaminase activity of whole broken cell preparations of lymphocytes, or of fractions prepared by differential centrifugation of such preparations. This suggested a participation of the lymphocyte membrane in the expression of the hydrocortisone effects observed with intact cells. However, preincubation of lymphocytes from postpubertal rats with hydrocortisone yielded cell fractions prepared from such lymphocytes which now exhibited the augmentation of transaminase activity seen previously with addition of the steroid *in vitro* to intact lymphocytes from mature rats. The observations suggest that the influence of hydrocortisone on transaminase activity is exerted within the cell and is dependent on some degree of integrity of the protoplasmic architecture.

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EFFECT OF AMPHENONE UPON STEROIDOGENESIS IN THE ADRENAL CORTEX OF THE GOLDEN HAMSTER[1,2]

BERNARD H. MARKS, M. ALPERT AND FRED A. KRUGER

Departments of Physiological Chemistry, Anatomy and Medicine, Ohio State University, Columbus, Ohio

ABSTRACT

In the golden hamster, amphenone does not produce the marked adrenomegaly or increase in adrenal cholesterol content seen in other species. In the hamster, too, there is no effect of amphenone on corticoidogenesis as measured by urinary steroid excretion. Administration of amphenone to the hamster results in the excretion of a metabolite that interferes with the Zimmermann test for 17-ketosteroids. It is postulated that the hamster, and perhaps other species with lipid-poor adrenals, may not utilize cholesterol as an adrenal steroid precursor, but may condense small metabolic units directly to a C21 steroid.

IT HAS been known for some years that there are distinct morphological and histochemical differences in the adrenal cortices of different mammalian species. Though the mammalian adrenal cortex is characteristically a lipid-rich structure (1, 2), several species are exceptions to this concept. The mammalian adrenal should probably be subdivided into lipid-rich and lipid-poor groups, depending on the size, number, and distribution of lipid droplets in the cortical cells, and the presence or absence of a positive Schultz test for cholesterol in these cells.

Using these criteria, most common laboratory animals qualify as having lipid-rich adrenals. These include specifically the rat and the dog, the animals used most generally in physiological and histological studies on the adrenal cortex. In contrast, the golden hamster, *Mesocricetus auratus*, like some other hibernating mammals and some ruminants, has a distinctly different kind of adrenal cortex. It is consistently negative to the Schultz test, and contains few, if any, lipid droplets (3). Sudan black produces only a moderate diffuse background stain with a few droplets in the glomerulosa; the only intense lipid staining is produced with the Schiff reaction for plasmalogens, the Baker reaction for phospolipids, and the O_2-Schiff ("Secondary Schiff") reaction for unsaturated fatty acids (4, 5, 6). These

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findings suggest that the predominant lipid in the hamster adrenal cortex is phospholipid rather than cholesterol or neutral fat, and is probably structural rather than stored metabolic precursor (4).

The hamster is also unusual in that its adrenal gland exhibits an inverted sexual dimorphism, the absolute and relative weights of the male glands being greater. This difference is abolished by gonadectomy (7). It has been stated also that the adrenalectomized hamster is better maintained by progesterone than DCA (8).

It was felt that the above differences in the morphology and histophysiology of the hamster adrenal cortex indicated rather basic differences in the functional biochemical pathways between animals with lipid-rich and lipid-poor types of glands. Accordingly, it was decided to investigate these differences by studying, in the hamster, the effects of drugs that are known to interfere in steroidogenic pathways. One such drug is Amphenone-B,[3] a substance which has been demonstrated to cause marked enlargement of the thyroid and adrenal glands of the rat, and which also possesses some progestational activity (9). The effects of amphenone on the rat adrenal have been attributed to an interference with corticosteroid synthesis, so that the use of this drug in the hamster might be expected to give us some information about this same process in a lipid-poor adrenal.

METHOD OF STUDY

The animals used were male golden hamsters, Tumblebrook Farm strain, initial weight approximately 100 g. They were compared with male Wistar strain albino rats, weight approximately 180 g. All were maintained on regular laboratory chow and water *ad libitum*. Amphenone was administered intragastrically through a fine polyethylene catheter. It was dissolved in water in such concentration that the daily dose per animal, 200 mg/kg., could be given in 0.5 cc. of solution.

Cholesterol determinations were performed according to the method of Coleman and McPhee (10) modified for tissue use. Urinary 17-ketosteroids were determined by the Zimmermann reaction according to Klendshoj *et al.* (11). 17-hydroxycorticoids in urine were determined by the Porter-Silber reaction of Reddy *et al.* (12) with modification (13).

Animals were sacrificed by decapitation, the left adrenal removed, trimmed free of all superfluous tissue, weighed on a Roller-Smith torsion balance, then placed in a thick-walled 12 cc. conical centrifuge tube. In this vessel, the adrenal was ground with a few grains of sand, using a heavy glass rod as a pestle, then extracted with 3:1 (v/v) alcohol-ether. The cholesterol determination was done on an aliquot of this extract. The right adrenal gland was removed and placed in 10% formalin. After several days fixation, frozen sections were cut and stained by the Schultz method and with Sudan black B. The remainder of the gland was dehydrated and embedded in paraffin for routine staining with hematoxylin and eosin.

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[3] 3,3-bis(p-aminophenyl)-butanone-2-dihydrochloride, will be referred to subsequently as amphenone (25). It was generously provided by Dr. Roy Hertz, National Institutes of Health, and by E. W. Young, The Upjohn Company.

RESULTS

Effects of feeding amphenone to golden hamsters are summarized in Figure 1 and Table 1. For the sake of comparison, data on similarly treated groups of control and experimental rats are included. The data obtained on the rats is consistent with earlier reports on this species (14, 15). It is apparent that in contrast to the rat, the hamster shows an insignificant

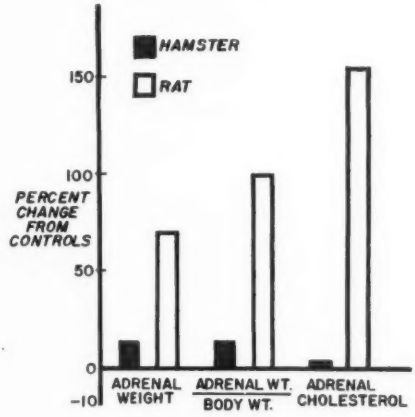

FIG. 1. Effects of amphenone upon the adrenal of the male hamster and rat.

change in absolute adrenal weight, in adrenal weight relative to total body weight, and in adrenal total cholesterol content. In addition, the histological preparations showed no Schultz-reactive material, and there was no change in the Sudan staining of the adrenals in the experimental animals, i.e., no accumulation of lipid droplets. Table 1 in addition demonstrates the reason for the lack of Schultz staining in the hamster—the adrenal total cholesterol content is only one-tenth that in the rat, and is below 0.5%, the level which some histologists consider necessary for the appearance of a positive histochemical reaction (Knouff, R. A.—personal communication).

TABLE 1. EFFECTS OF AMPHENONE UPON THE ADRENAL OF THE MALE HAMSTER AND RAT

Treatment	Number	Left adrenal weight, mg.	A/B ratio*	Adrenal cholesterol per cent
Control hamster	5	10.4 ± .9	12.1 ± 1.0	.29 ± .03
Amphenone† hamster	7	11.7 ± 1.3	13.7 ± 1.7	.30 ± .04
Control rat	4	19.8 ± 3.7	9.1 ± 1.8	3.20 ± .67
Amphenone† rat	4	33.5 ± 8.4	18.1 ± 4.8	8.12 ± 1.09

Mean ± S.D.
* Left adrenal wt. in mg./body wt. in g. × 100.
† Amphenone 200 mg./kg./day P.O. × 10.

In order to further characterize the apparent lack of amphenone effect in the golden hamster, a group of males was castrated, and upon recovery from this procedure, the urinary 17-ketosteroid excretion was studied before and after amphenone treatment. Results are shown in Table 2 in terms of micrograms of 17-ketosteroid per 24 hour urine sample.

The control castrate male hamster excretes approximately 38 μg. 17-ketosteroid per day. We were surprised to see the amphenone-treated animals excreting about 2½ times that much, but it was quickly realized that this must represent the presence in the urine of an amphenone metabolite. That this could not possibly be a false-positive test from amphenone itself was shown by a control reaction with a sample of the pure drug. The slight color produced in the Zimmermann reaction by the total daily dose of pure amphenone could only account for a small percentage of the value actually found.

TABLE 2. EFFECT OF AMPHENONE UPON URINARY 17-KETOSTEROID OF THE CASTRATE MALE HAMSTER

Treatment	Number	Urinary 17 ketosteroid μg/24 hours
Control	9	38 ± 7.3
Amphenone*	4	94 ± 23.5
Amphenone-adrenalectomized	5	62 ± 13.8
Adrenalectomized	1	6

Mean ± S.D.
* Amphenone 200 mg./kg./day P.O.

Proof of the excretion of this postulated amphenone metabolite was obtained by adrenalectomizing the castrate male hamsters which were being treated with the drug. Three days after adrenalectomy these animals were still excreting 62 μg. of apparent 17-ketosteroid, at a time when there was no known source of this steroid. That all of this material represents a non-steroidal chromogen was shown by discontinuing the amphenone treatment, after which, in the instance in which we could keep the animal alive, the urinary 17-ketosteroid fell to 6 μg. per day—actually about the lower limit of sensitivity of the method.

Subsequent investigation of this chromogen resulting from amphenone treatment indicates that it produces a broad band of absorption in the visible range without the peak at 510 mμ which characterizes the 17-ketosteroids. Preliminary attempts at separating and identifying this chromogen with paper chromatography have not yet been successful.

Since Hertz (9) reports a fall in the level of glycogenic steroid production in the amphenone-treated rat, a study of 17-hydroxycorticoid excretion in the hamster was performed. Amphenone does not interfere in the Porter-Silber reaction. The drug was administered to 3 castrate males, dose 200 mg./kg./day. Control animals excreted .16 mg. of 17-hydroxycorticoids per 24 hours. After five days of treatment, no effect upon steroid excretion

was seen (.14 mg./day excreted), after ten days some augmentation might have occurred (.31 mg./day excreted). Certainly there was no inhibition of steroidogenesis.

DISCUSSION

It is probably safe to assume that absorption of amphenone takes place from the gastro-intestinal tract of the golden hamster, though the feces were not analyzed for their content of the drug. Since, in some female hamsters that were treated, definite endocrine effects were seen in the enlargement of the uterine horns, an effective blood level of the drug must obtain in this sex. Furthermore, the excretion of a metabolite of amphenone in the urine is an indication of absorption. We do not, however, know the site nor the extent of the degradation of this agent, nor the duration of the effective blood levels. The metabolite excreted by the hamster seems to behave similarly to that described as occurring in the urine of man (18). From studies on the intact and perfused adrenal (16, 17) it is clear that amphenone itself has the effect upon the gland, although it has not been established whether or not the metabolic products are also active.

It seems clear that the reason for the adrenal enlargement following amphenone treatment of various species is an inhibition of steroid secretion followed by a compensatory increase of corticotrophin release by the anterior pituitary. The inhibition of corticoid secretion has been demonstrated in the dog (19), the calf (17), and man (18, 20, 21). The corticotrophin requirement was demonstrated in the rat by Hertz, who showed that hypophysectomy (9) or cortisone administration (22) would prevent adrenomegaly. The marked accumulation of cholesterol which occurs in the adrenal of the amphenone-treated rat is interpreted as indicating a block in the conversion of precursor cholesterol to corticosteroid. This interpretation is strengthened by the direct demonstration that amphenone inhibits numerous of the pathways of steroid synthesis in the adrenal in doses which do not materially affect the oxygen consumption of the tissue (17). Amphenone is then a specific inhibitor of steroid synthesis and not a nonspecific toxin. Any species that depends upon the availability of cholesterol as a precursor of adrenal steroids should show cholesterol accumulation in the adrenals of amphenone-treated animals. Conversely, if no cholesterol accumulation occurs, this would indicate a difference in the metabolic pathway for steroid synthesis.

In every other species in which there has been a study of the steroid excretion following amphenone treatment, a marked reduction in the output of one or more steroids has been noted. At the same time, presumably, there would have been accumulation of cholesterol in the adrenal. This correlates well with the accepted view of amphenone action and the present view of steroid synthesis in the adrenal cortex (23).

In the case of the hamster, however, there is clearly no piling up of

cholesterol, no inhibition of steroidogenesis (although we have not examined aldosterone secretion), and consequently no adrenal hypertrophy. In order to account for this departure from the expected behavior, it may be necessary to postulate that this species, and perhaps others with lipid-poor adrenals, does not utilize the usual method of steroid synthesis. Instead, it might condense short-chain units directly to a C21 rather than a C27 compound (cholesterol), and thus not require some of the metabolic steps which amphenone inhibits. This would accommodate many of the observations on the histophysiology of the hamster adrenal cortex. The initial low cholesterol, rather poorly responsive to stress or ACTH (4, 5), and the failure to obtain changes with amphenone are all explained by this alternative to the generally accepted steroidogenic scheme. At present, the possibility of qualitative and quantitative differences in the detoxification of amphenone by the hamster cannot be completely discarded. Nevertheless, we feel that the most reasonable hypothesis explaining our findings is a basic biochemical difference in the hamster's adrenal cortical metabolism.

Support for the postulate which we offer could be obtained with the use of labelled substrates in the hamster. It has already been demonstrated, though, that in cell-free preparations of hog adrenal there is incorporation of labelled acetate directly into adrenal corticoids, without any labelling of the adrenal cholesterol pool (24). We suggest that this process, which in other species occurs only in acellular preparations, may be the predominant one in the intact adrenal gland of the hamster.

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METABOLISM OF CORTISOL IN NORMAL AND "STRESSED" DOGS[1]

KRISTEN B. EIK-NES AND LEO T. SAMUELS

Department of Biological Chemistry, University of Utah, College of Medicine, Salt Lake City, Utah

ABSTRACT

The removal rate of intravenously injected cortisol has been determined in normal and "stressed" dogs. Normal or decreased removal rates of cortisol were found in all "stress" conditions investigated. Mechanisms other than increased "utilization" of adrenal steroids might therefore be responsible for augmented ACTH secretion in conditions of "stress." Various theories which might explain why intravenously given cortisol is removed more slowly in severely "stressed" dogs are discussed.

A CENTRAL point in the physiology of the adrenal cortex is its function during conditions of "stressful" nature. It appears from the literature that many such conditions are associated with an increase in plasma levels of 17-hydroxycorticosteroids (17-OHCS) (1, 2). Little is known, however, concerning the immediate causes of increases. It has been postulated (3) that during "stress," the metabolism or "utilization" of adrenal steroids may be increased. Since this would result in a reduction of the concentration of adrenal steroids in the blood, this might constitute a stimulus for increased secretion of ACTH by the anterior pituitary gland. If this theory were correct, one would expect that cortisol given intravenously to predominantly cortisol-producing species (4) would be removed at an accelerated rate when the animal was exposed to "stressful" environments.

This work deals with the removal rates of cortisol in dogs exposed to different changes in body conditions.

EXPERIMENTAL

Mongrel dogs of both sexes were used. The animals were obtained from the Salt Lake City dog pound and kept in the University dog kennels for 4–5 days prior to experimentation. Only one experiment was conducted on each dog and all were commenced between 8 and 9 A.M.

Five different conditions were used:

A. Removal of 30 ml. blood five times over a 4- to 6-hour interval in unanesthetized dogs previously accustomed to venesection by repeated withdrawals over a period of four to six months.

B. Intravenous administration of 35 mg. sodium pentobarbital per kg. body weight

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and cannulation of the femoral artery (5). A 30 ml. blood sample was withdrawn from these animals five times over a 4- to 6-hour interval.

C. After anesthesia and cannulation, the animal was placed in a walk-in cold room with a floor temperature of about +5° C. Removal of blood was done as in B.

D. After anesthesia and cannulation, the animal was exposed to an oscillating hot lamp. Skin burns were prevented by wrapping the animal in cheese cloth. Removal of blood was done as in B.

E. After cannulation and anesthesia, the animal was given 20γ of bacterial polysaccharide (Piromen) per kg. body weight intravenously. Removal of blood was done as in B.

In the conscious control dogs the first blood samples were withdrawn 10–15 minutes after the animals had been taken to the laboratory. Throughout the experiments they were kept caged in the intervals between the drawing of blood.

Plasma 17-OHCS were determined by the method of Nelson and Samuels (6). The metabolism of cortisol was investigated by giving 1 mg. per kg. body weight intravenously dissolved in 5% ethanol-95% isotonic saline. Blood samples were obtained from the animals 1, 2, 3 and 4 hours following cortisol administration.

Rectal temperature was measured by a Foxboro automatic temperature recorder.

The following drugs were administered intravenously: sodium pentobarbital (Abbott lot No. 3778), cortisol (Upjohn lot No. 2582-ARH-64), and bacterial mucopolysaccharide (Piromen, Baxter lot No. L 10).

RESULTS

All the "stress" conditions tried were associated with an increase in plasma 17-OHCS levels except where normal dogs were exposed only to the "stress" of frequent removal of blood (Table 1). The change in dogs with low body temperature (Condition C) was not as great as in animals with high body temperature (Condition D, Table 1 and Fig. 1). The mortality rate was high among animals in "stress" categories C and E. No animal was able to survive "stress" condition C for more than 10–12 hours and

TABLE 1. MEAN PLASMA LEVELS OF 17-OHCS IN DOGS EXPOSED TO VARIOUS "STRESS" CONDITIONS

Condition	Number of animals	17-OHCS μg./100 ml. plasma			
		Hours after stress			
		0.1–0.2	2	4	6
A	11	4 (2–7)*	3 (1–5)	2 (1–3)	2 (1–3)
B	15	5 (2–12)	8 (4–12)	10 (4–16)	12 (9–15)
C	6	5 (3–6)	8 (4–11)	8 (7–13)	9 (7–13)
D	10	6 (3–8)	21 (14–29)	21 (9–32)	20 (10–30)
E	13	6 (3–10)	20 (12–32)	24 (14–35)	25 (15–39)

* Range.

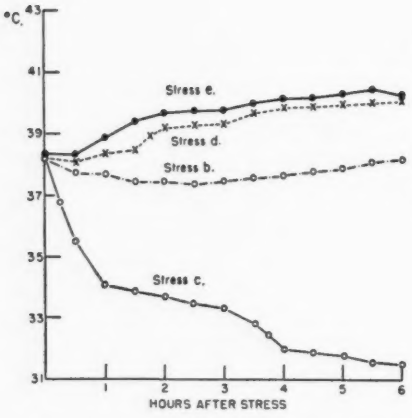

FIG. 1. Average rectal temperature in dogs subjected to different forms of "stress."

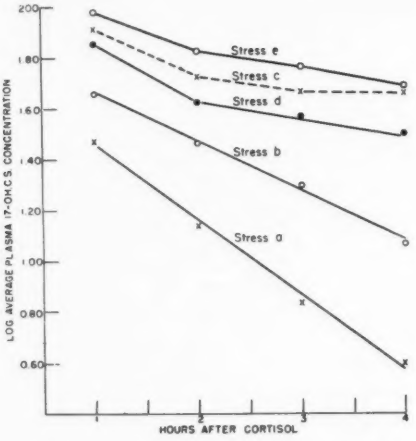

FIG. 2. Average plasma 17-OHCS levels following the intravenous administration of 1 mg. cortisol/kg. in dogs subjected to various forms of "stress." The average plasma 17-OHCS levels are plotted logarithmically against time following cortisol injection.

36% of the animals subjected to "stress" condition E died. Maximal death rate was observed 12–24 hours after the administration of the polysaccharide. No death occurred in conditions A and B. All dogs exposed to stress condition C had a very slow flow of blood through the cannula placed in the femoral artery.

Between 2 and 4 hours after anesthesia, cortisol was given intravenously to groups B–E (Table 2). Nine of the animals in group B received cortisol as soon as the operative procedure was completed, but since no significant difference between these and the group injected later was observed the data have been pooled. Considerable spread in plasma levels of 17-OHCS was seen. If the logarithms of the average plasma 17-OHCS concentrations after cortisol administration are plotted against time after administration, the points fit a straight line in conditions A and B (Fig. 2). This was not true for conditions C, D and E.

DISCUSSION

Under the conditions studied, it appears that in the trained normal dog the removal of 30 ml. blood every second hour for 6 hours (Condition A) did not affect plasma 17-OHCS levels. This most certainly is an unpleasant experience for the animals since the majority reacted to the needle puncture. Whether or not the condition qualifies as a "stress" is not for us to decide, since the term "stress" remains as a rather loose biological definition.

Since the experiments were started between 8 and 9 A.M., the fall in plasma 17-OHCS in condition A could reflect a diurnal variation of these steroids. High plasma concentrations of 17-OHCS in the morning, falling off over the late morning and early afternoon hours have been observed in man (7). These data indicate that this is also the case in the dog; the diurnal fluctuation of plasma 17-OHCS is of much smaller magnitude than in man, but similar in proportion to concentration.

By different and indirect techniques for the measurement of adrenocortical function, the "stress" of heat (8), the "stress" of cold (9), the "stress" of operative trauma (1) and the "stress" associated with the administration of pyrogens (10) has been said to be connected with hypercorticism

TABLE 2. MEAN PLASMA LEVELS OF 17-OHCS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 1 MG. CORTISOL/KG. IN DOGS SUBJECTED TO VARIOUS FORMS OF "STRESS"

Condition	Number of animals	Rectal temp. before F ° C	17-OHCS before F γ/100 ml.	17-OHCS, μg./100 ml. plasma			
				Hours after F			
				1	2	3	4
A	10		3 (2–4)*	30 (20–35)	14 (10–17)	7 (4–10)	4 (2–6)
B	14	37.6 (37–38.6)	7 (3–11)	46 (33–65)	30 (21–34)	20 (14–34)	12 (6–22)
C	5	32.7 (32–34)	12 (9–15)	83 (63–123)	53 (38–62)	46 (39–56)	46 (37–50)
D	5	40.2 (39.9–40.6)	24 (16–28)	74 (46–86)	43 (19–78)	39 (20–78)	32 (15–57)
E	6	40.5 (40–41.3)	25 (14–39)	96 (75–117)	67 (57–83)	60 (50–85)	45 (39–54)

* Range.

in different animals. Using circulating levels of 17-OHCS these findings have been substantiated in the present investigation.

The "stress" response of anesthetized animals exposed to cold is slight compared to the other "stress" responses seen. This has been reported independently from two laboratories (11, 12). Further, it has been found that the plasma 17-OHCS levels of anesthetized animals with low rectal temperatures do not increase following ACTH injection (12) whereas those of anesthetized dogs with normal rectal temperatures will (5). The data suggest that when body temperature is lowered, the rate of synthesis and release of steroids by the adrenal gland, like other chemical reactions, is reduced. If so, ACTH may be secreted at an increased rate during this form of "stress" but the adrenocortical cells are rendered refractory to ACTH action.

Very early following anesthesia and cannulation increased adrenocortical activity is apparent. Within the first 15 minutes the mean levels of 17-

OHCS are higher than those of the controls in all the groups exposed to these procedures. This "stress" response timewise is in good agreement with the data of Sayers and Burks (13) covering ACTH levels in rat plasma following ether anesthesia.

"Stress" conditions B–E did not accelerate the removal rate of intravenously given cortisol; it was either normal or decreased. The decay rates of cortisol in the severe "stress" conditions associated with a high mortality rate are similar to those found in the dying human subject (14). In the severely "stressed" dog, therefore, one may be dealing with the same parameters of cortisol metabolism as seen in the dying man. In the latter case it was clearly demonstrated that tetrahydrocortisone was removed at the same rate as in healthy subjects. The first reduction reaction appeared to be the rate limiting step in the metabolism of cortisol in man (15). That such a reaction is taking place in the dog's liver is evident from the work of Axelrod and Miller who isolated and identified saturated 3-hydroxylated compounds following hepatic infusion of cortisol in the dog (16). Impairment of liver function has been seen following the administration of pyrogens with the subsequent development of fever (17), and decreased liver capacity during the "stress" of surgical operation has been observed in man (18). The present data indicating a change in cortisol metabolism in life-threatening "stress" conditions are probably, therefore, a reflection of reduced hepatic activity.

A slow flow of arterial blood was seen in all animals having low body temperatures. Deceleration in arterial blood flow in this condition may also have affected the blood flow through the liver. If so, this could in part explain why dogs with low body temperature metabolize intravenously given cortisol more slowly than the normal animal. Englert *et al.* (19) have recently demonstrated that the blood flow through the liver enters into the picture of cortisol metabolism in the dog since animals with a devascularized liver by surgical means exhibited a significantly greater half life of intravenously given cortisol than normal animals or dogs with increased hepatic blood flow. On the other hand, the low body temperatures in the cold "stressed" animals may be too low to be optimal for the function of the hepatic enzymes involved in the reduction of cortisol. Hence, both the function of the liver cells and the flow of blood through the liver are determining factors in cortisol metabolism in the dog.

Finally, the difference in the removal rate of cortisol between normal dogs and "stressed" dogs may be due to an increased ability of the peripheral plasma of the latter to combine loosely with cortisol. Several groups of workers (20, 21, 22) have observed that cortisol may combine with certain plasma proteins, lipoproteins or glucoproteins. The nature of such a combination may involve weak forces and may be of the same character as described by Eik-Nes *et al.* (20). It is interesting in this respect to note that Chanutin and Gjessing (23) found a fall in serum albumin and an in-

crease in a serum α-globulin in dogs subjected to unspecific "stress." Further work by this group (24) showed that this substance, found only in "stressed" dogs, was rich in lipids. Whether such a protein functions *in vivo* as a carrier of adrenal steroids is not known. This possibility warrants further investigation, however.

Our observation of either a normal or a decreased cortisol removal rate in "stress" conditions seems to agree with current data on this topic. Done and Kelley (25) report a normal or decelerated rate of cortisol metabolism in subjects suffering from different diseases. Ulrich and Long (26) conclude from their experiments with cortisol-4-C^{14} injections in "stressed" rats that the concept of increased peripheral "utilization" of adrenocortical hormones during "stress" was not proved. Firschein *et al.* (27) observed that cortisol-4-C^{14} is metabolized at a slower rate in shocked rats than in normal control animals. Normal or decreased metabolism of intravenously given cortisol has recently been observed in man following the "stress" of a surgical operation (28).

In view of the experimental findings one should look for factors other than increased "utilization" of adrenal steroids initiating, and probably maintaining, an increased secretion of ACTH in conditions of "stress."

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SYNERGISM OF ESTROGEN AND PROGESTERONE ON MAMMARY GLAND GROWTH IN GUINEA PIGS[1]

THOMAS C. SMITH AND BRITTA RICHTERICH

Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts

ABSTRACT

Guinea pigs were treated for varying periods of time with a range of doses of estradiol benzoate and/or progesterone. Typically, the steroids were injected subcutaneously in separate sites three times a week for four or eight weeks. Growth was assessed histologically in all experiments. In animals given combinations of estrogen and progesterone, measurements of nucleic acids, total nitrogen, per cent dry and defatted-dry weight, and slice respiration of mammary tissue supplemented the histological approach.

The amount of lobulo-alveolar growth attained with estrogen alone depended upon both the dosage and length of treatment. Glands from males showed less growth after estrogen than those from females, while those from intact females were larger than those from castrated females. No dose of estrogen was found which elicited ductal proliferation without at the same time causing some lobular growth, but the dose was limited so that the synergistic action of progesterone could be demonstrated. Progesterone alone induced no growth, but when added to the estrogen treatments, enhanced lobulo-alveolar growth. Augmentation of parenchymal growth was paralleled by increases in percentage dry weight, and especially percentage defatted-dry weight of the organ. Total mammary weight proved an inaccurate index of hormonal stimulation.

Combinations of estrogen and progesterone brought about growth of mammary glands with greater concentrations of DNA and RNA, increased respiration, and slightly greater concentrations of total nitrogen than obtained with estrogen alone. The concentration of these constituents and respiratory activity was also dependent upon the doses of the hormones. Mammary glands developed experimentally showed lower concentrations of nucleic acids and less respiratory activity than those of late pregnancy. The results are believed to show that progesterone synergizes with estrogen in eliciting lobulo-alveolar growth of the mammary glands in guinea pigs in a manner similar to that established for other species, in spite of earlier negative reports.

THE guinea pig has been reported to be one of those species whose mammary glands respond to estrogen with complete growth of both the lobulo-alveolar and ductal components (1, 2). Progesterone has, therefore, been considered relatively unimportant in the experimental induction of mammary growth in this species. This impression has been based

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mostly upon early exploratory studies (3, 4). Even so, perusal of these reports shows that evidence of a role for progesterone was at hand, being equivocal in some cases and overlooked in others. It therefore appeared worthwhile reinvestigating the problem of the relative importance of estrogen and progesterone in mammary growth in the guinea pig, on the presumption that the interaction of the two hormones upon the mammary gland, as shown in histological studies on the mouse (5), rabbit (6, 7) and rat (8), would also apply to the guinea pig.

The results presented here in detail have been reported in a preliminary note (9). The type of growth attained in our experiments was closely related to the nature of the hormonal stimulation; that elicited by a combination of estrogen and progesterone most closely approximating that achieved during late pregnancy in this species. Growth of the gland has been assessed in these studies both histologically and biochemically. Two biochemical criteria, the content of nucleic acids and nitrogen, and the oxidative activity were selected, and comparisons were made with values previously reported for the gland of the pregnant or lactating guinea pig (10).

MATERIALS AND METHODS

All guinea pigs were fed *ad libitum* a diet of Ralston-Purina chow checkers, supplemented with cabbage. Surgical operations were conducted with clean, but not aseptic technique. All hormones were administered three times a week by subcutaneous injection in 0.1 ml. sesame oil, control animals receiving an equal volume of the vehicle.[2] At autopsy, two to three days after the last injection, the animals were stunned and exsanguinated and the mammary glands rapidly removed to either the fixative or ice-cold Kreb's Ringer-phosphate (cf. 11).

Glands were removed *in toto* to Bouin's fixative in the first experiments. After the fixative was extracted from the gland with alkaline 70% ethanol, the gland was trimmed of adhering fat and connective tissue in order to visualize its general outline. From a thick cross-section taken in the middle of the gland, 8μ sections were prepared, following the plane of the cross-section in cutting, and stained with hematoxylin and eosin.

For the histochemical studies, the whole trimmed glands were weighed, pieces were fixed in neutral buffered formalin, processed, and stained with the McManus-Hotchkiss method (cf. 12). The growth of ducts, buds, and alveoli, the amount of stroma, and the secretory activity were rated from the histological sections. The remaining tissue was used for the preparation of slices with the Stadie-Riggs microtome and homogenates with a conical ground-glass homogenizer, according to the procedure previously described (10). The respiration of slices suspended in Ringer, phosphate, pH 7.4, without substrate or with succinate, 20 mM/L., was measured in air at 37.5° C, for one hour with a Warburg respirometer. Respiration was calculated as μL O_2 uptake per hour per mg. fresh, dry, or defatted-dry weight or total nitrogen (TN), deoxynucleic acid (DNA), or ribonucleic acid (RNA).

Homogenates were analyzed for total nitrogen (TN) after digestion in 18N H_2SO_4 for 45 minutes, treatment with superoxol, followed by Nesslerization and reading in a

[2] We wish to thank Ciba Pharmaceutical Products, Inc. for the estradiol benzoate and the Schering Corporation for the progesterone used in these studies.

Klett colorimeter with a #44 filter. Comparisons were made with colors developed with standard solutions of $(NH_4)_2SO_4$ (range, 50–250 mg. nitrogen). Nucleic acids were determined, using the trichloracetic acid fractionation of Schneider (13), and the colorimetric reactions of diphenylamine for desoxyribose (14) and of orcinol for ribose (15). All values were referred to simultaneously-run standard solutions of fish sperm desoxyribonucleic acid (DNA) and yeast ribonucleic acid (RNA). The concentrations of these constituents were expressed as μg. per mg. fresh, dry, or defatted-dry weight. Dry weight and defatted-dry-weight were obtained on both slices and homogenates by methods described in an earlier paper (10).

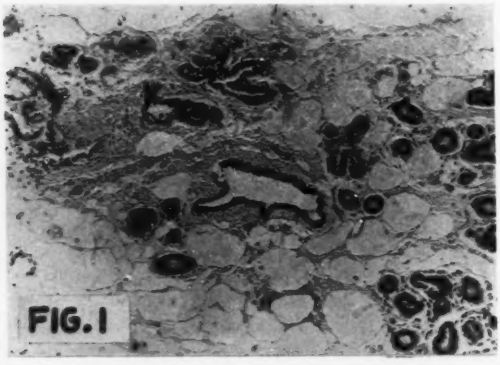
Twenty-eight male guinea pigs were treated for two or four weeks in a preliminary experiment with 0.25 to 16 μg. estradiol benzoate per injection three times a week. The results showed that two weeks was insufficient time to stimulate much tissue growth regardless of dose. Less than 2.0 μg. per injection for four weeks produced ductal stimulation with limited lobulation. In a further experiment, 24 males and 24 nulliparous females were injected with 0.25 to 4.0 μg. per injection three times a week, half of both sexes having been castrated several weeks prior to treatment. These experiments indicated the best range of estrogen doses for future experiments. They further showed that, not only was the mammary gland of males much less responsive to these doses of estrogen, but also that the presence of the ovary in the female resulted in greatly enhanced glandular thickness and lobulation.

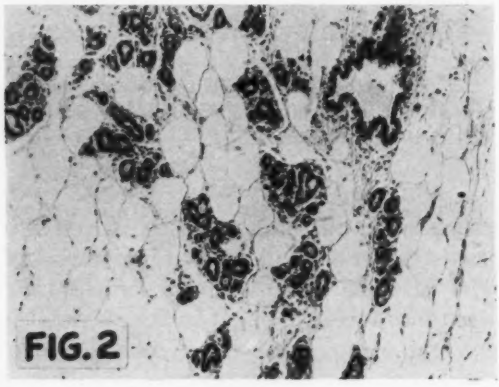
RESULTS

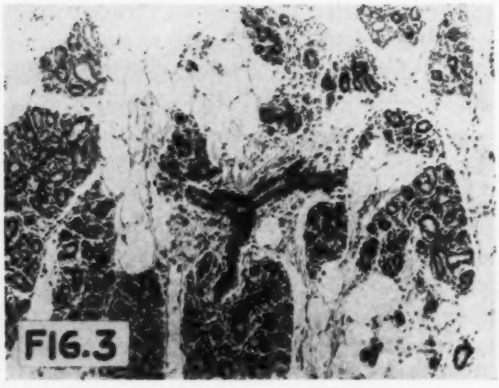
Female guinea pigs were bilaterally ovariectomized at least one month prior to the initiation of treatment. Two dozen were injected with 0.25 to 1.0 μg. estradiol benzoate and 0.5–5.0 mg. progesterone, alone, and in combination, three times a week for eight weeks, and another group treated similarly for only four weeks. Since the number of animals on any one dose combination was small, the results have been grouped into dosage classes to simplify presentation.

Estrogen alone, given for either four or eight weeks, induced the formation of lobules as well as branching and proliferation of ducts. The density and size of lobular elements was directly related to the dose of estrogen (Figs. 1–3). Progesterone alone induced no growth. The addition of progesterone to estrogen treatment, however, resulted in greatly enhanced lobulo-alveolar growth, comparable to that of late pregnancy (Figs. 4–6). The amount of secretory material in the lumina of ducts and alveoli was also more pronounced in the mammary glands of guinea pigs receiving the higher doses of estrogen and progesterone than in those receiving any dose of estrogen alone. Augmentation of lobulo-alveolar growth was most marked in animals receiving either 48 or 120 mg. progesterone (total dose), but was detectable when compared to any dose of estrogen alone.

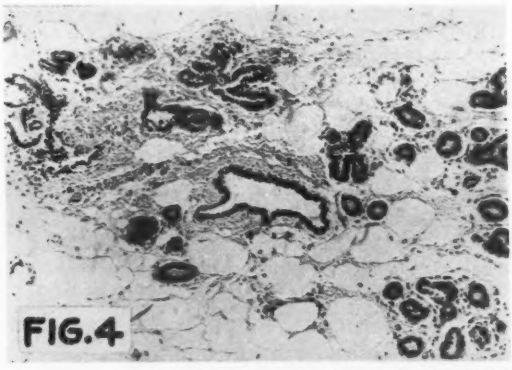
Total relative mammary weight was an inadequate measure of mammary growth, due in part of the difficulties in delineating the glandular material from surrounding tissue. Percentage dry weight, and especially percentage defatted-dry weight, while greater in glands from animals treated with estrogen than with progesterone, was greatest with those from animals given both hormones together (Table 1). Dry and defatted-dry weight

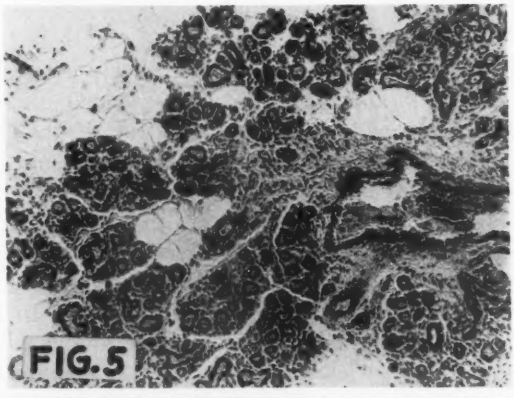






FIGS. 1–3. Mammary glands of ovariectomized guinea pigs treated with estrogen three times a week for eight weeks, showing graded effect of dosage on density and size of lobules. Total dose of estradiol benzoate: Fig. 1, 6 μg.; Fig. 2, 12 μg.; Fig. 3, 24 μg. ×128.





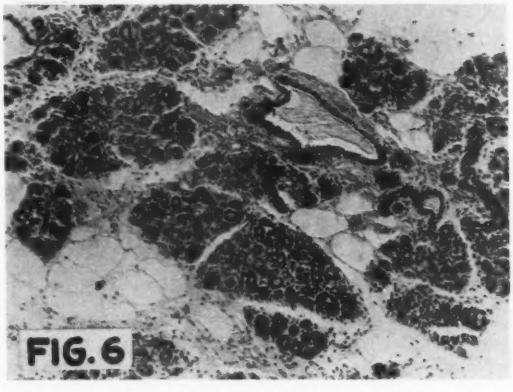


FIG. 4. Guinea pig 4–18, treated with a total dose of 6 μg. estradiol benzoate for 8 weeks: a few small, scattered lobules. ×128.

FIG. 5. Guinea pig 4–6, treated with total doses of 6 μg. estradiol benzoate and 120 mg. progesterone for 8 weeks: potentiation of lobular-alveolar growth. ×128.

FIG. 6. Mammary gland from guinea pig in terminal week of pregnancy. ×128.

TABLE 1. RELATIVE WEIGHT AND PER CENT DRY AND DEFATTED-DRY WEIGHT OF MAMMARY GLANDS AFTER VARIOUS HORMONAL TREATMENTS

Group	Mam. gland wt. / B. wt. ×100	Per cent dry weight	Per cent defatted-dry weight	Number of animals
Estrogen	0.26±0.03	15.3±2.4	4.3±1.0	6
Per cent difference	23	25	47	
Estrogen and progesterone	0.32±0.05	19.1±1.8	6.3±0.9	15

Ovariectomized guinea pigs injected three times a week for eight weeks with total dose of 6–24 μg. estradiol benzoate and/or 12–120 mg. progesterone (see text for further details).

$$\text{Standard Error} = \sqrt{\frac{\Sigma(X)^2 - \frac{(\Sigma X)^2}{n}}{n(n-1)}}$$

also showed increments with increase in dose of estrogen (data not shown). On the other hand, with any constant dose of estrogen, increasing the dose of progesterone did not lead to any change in the weight properties of the organ. Since progesterone alone had little effect on eliciting growth, sufficient material was not available from such animals to obtain reliable data on tissue composition.

Combinations of estrogen and progesterone bring about growth of mammary glands with strikingly greater concentrations of both deoxynucleic acid (DNA) and ribonucleic acid (RNA), but only slightly greater concentrations of total nitrogen (TN) than those of guinea pigs treated with estrogen alone (Table 2), whether the data are calculated on the basis of defatted-dry weight, dry weight, or fresh weight. Both the endogenous and the succinate-stimulated respiration of glands from guinea pigs treated with combinations of estrogen and progesterone is greater than that from guinea pigs treated with estrogen alone, when expressed on the basis of fresh weight (Table 3). This increment merely keeps pace with the increase in parenchymal mass, since the differences are minimal when the results are related to either defatted-dry weight or nucleic acid content. On the other hand, respiratory activity of glands from estrogen-treated animals was greater than that of progesterone-treated animals when calculated on both a fresh weight and DNA basis.

TABLE 2. NUCLEIC ACIDS AND TOTAL NITROGEN AFTER VARIOUS HORMONAL TREATMENTS

Group	Concentration, μg./mg. DDW			Number of animals
	DNA	RNA	TN	
Estrogen	63.2± 5.7	8.6±2.9	113.8± 9.5	6
Per cent difference	105	154	15	
Estrogen and progesterone	118.2±14.9	17.8±1.8	130.8±12.1	15

Ovariectomized guinea pigs injected three times a week for eight weeks with total dose of 6–24 μg. estradiol benzoate and/or 12–120 mg. progesterone (see text for further details).

TABLE 3. MAMMARY GLAND RESPIRATION AFTER VARIOUS HORMONAL TREATMENTS

Group	Endogenous Q_{O_2} FW	Succinate Q_{O_2} FW	Number of animals
Progesterone	0.05	0.13	4
Estrogen	0.10 ± 0.02	0.23 ± 0.03	6
Per cent difference	140	87	
Estrogen and progesterone	0.24 ± 0.02	0.43 ± 0.03	16

Ovariectomized guinea pigs injected three times a week for eight weeks with total dose of 6–24 μg. estradiol benzoate and/or 12–120 mg. progesterone (see text for further details).

An indication of enhanced mammary response to increments in the dosage of hormones was seen, although there were few animals per dose combination. With estrogen alone, elevation of dose led to greater concentrations of both nucleic acids and total nitrogen, and in both endogenous and succinate-stimulated respiration per milligram fresh weight. Similarly, on a constant dose of estrogen, increase in progesterone dose also brought about increments in these constituents. In this regard it appeared that 48 mg. progesterone excreted a maximal effect with 6–12 μg. estrogen, while 120 mg. progesterone showed its greatest action with 24 μg. estrogen.

When the weight and biochemical properties of the experimentally-induced glands were compared to those previously determined for the guinea pig during late pregnancy, absolute and relative mammary weight, per cent dry weight, and nitrogen concentration are similar. Desoxynucleic acid and endogenous respiration does not, however, approach that of the glands of the gravid guinea pig. These inadequacies might have been due to either a missing hormonal principle or improper dosage. Addition of testosterone, 1 mg. per injection, to treatment with either estrogen alone or a combination of estrogen and progesterone for eight weeks does not augment, but antagonizes the actions of the female steroids in nearly all respects. Doubling the frequency of injections of the highest doses of estrogen and progesterone resulted in greater relative mammary weight, per cent dry weight, and DNA content after four weeks. After eight weeks, there was a slightly greater mammary weight and definite increments in per cent defatted-dry weight, concentration of nucleic acids and nitrogen per mg. fresh or dry weight, and in endogenous respiration. Nonetheless, DNA content and respiration remained less than in glands from pregnant animals. Doubling the length of treatment from four to eight weeks with 0.25–1.0 μg. estrogen and 5 mg. progesterone per injection brought about an increase in only the total and relative mammary weight.

DISCUSSION

The relative ease with which lobuli may be induced to grow with estrogen in the guinea pig is a qualitative species difference, since consistent and normal growth of these structures does not ensue in the rat (8) or mouse (5) even when very high doses of estrogen are given. A dose or range of

doses for estradiol benzoate cannot be stated from the present experiments because the effects of the estrogen were purposely limited to facilitate demonstration of a role for progesterone. Unresolved discrepancies still exist in the literature regarding the relative potencies of two other estrogens, estrone and diethylstilbestrol, in stimulating teat and mammary growth in this species (3, 16, 17). Increase in teat length was, in fact, the first end-point used in an attempt to demonstrate a dose-response effect with estrone (3). At about the same time, growth of the teat and of the mammary gland were also reported to be correlated (18). Finally, the quality, as well as the extent of growth, is markedly dependent upon the dose of estrogen (16).

Early observations on mammary changes during the estrus cycle in guinea pigs indicated that in pubertal, nulliparous animals, there was a marked stimulation of the duct system at the approach of estrus, but that during the luteal phase, regression occurred (19, 20). Alveoli were never seen normally, but if deciduomata were produced 4 to 8 days after ovulation, the succeeding ovulation was often delayed and the cycle prolonged, leading to greater mammary proliferation (21). Hysterectomy was later found to bring about the same sequence of events, resulting in even greater stimulation of mammary growth (22).

These experiments seem to indicate, in retrospect, that if the luteal phase be sufficiently prolonged, progesterone or a congener might exert an effect. Although progesterone alone exerted no detectable effect in the present experiments, very small amounts synergized with estrogen on mammary lobulo-alveolar growth. The minimal daily dose of progesterone per 100 gm. body weight necessary for lobulation in guinea pigs (15 μg.) is far less than that required in rats (2000 μg.; 8), slightly less than that needed in mice (33 μg.; 5) and about the same as that reported in rabbits (16–17 μg.; 6).

Earlier reports (3, 4) of the inability of progesterone, even in combination with estrogen, to influence mammary growth in the guinea pig, was likely due to either insufficient progesterone or to masking with excess estrogen. In a recently published study, however, Benson, *et al.* (23) have amassed evidence that progesterone shows a synergistic action with estrogen on lobulation on the guinea pig. These authors also note that complete mammary development does not occur with estrogen alone, as previously reported (3, 4), although considerable growth of lobules with normal structure was obtained. Some of this work has also appeared in preliminary reports (24, 25).

Mammary glands of male guinea pigs, while responding qualitatively the same as those of castrated females, are not as sensitive to the lower doses of estrogen. Previous suggestions of this sex difference have been reported (3, 17), but seem to have been overlooked. It may be that endogenous androgen contributes to this decreased responsiveness, unless the

estrogen dose is sufficient to inhibit gonadotropin and endogenous androgen production. Certainly, a direct antagonism of endogenous androgen to the action of the ovarian steroids on the mammary glands is probable, judging from the inhibitory effects seen in the present experiments with exogenous testosterone.

Little previous work has appeared on the biochemical properties of hormone-stimulated mammary gland of the guinea pig, although a study outlining some of the metabolic properties during pregnancy and lactation has been reported (10). In one study (26), guinea pigs were injected with estrogen and progesterone for three weeks. The mammary glands showed at autopsy succinoxidase activity and coeneyzme A concentration comparable to that of the pregnant gland (27), but further treatment with prolactin or growth hormone alone or in combination with ACTH and dl-thyroxine failed to increase these activities to levels found in the lactating gland. The dose of estradiol, 250 μg. daily, was sufficiently high to elicit nearly complete ductal and alveolar growth by itself, making the contribution of progesterone, 1.0 mg. daily, negligible. In our experiments neither the oxidative activity nor the concentrations of deoxynucleic acid of the hormonally-stimulated glands attained that of glands from pregnant animals.

Quantitatively, nucleic acid concentrations and oxidative metabolism appear to be the most sensitive biochemical criteria of lobular development. Both were augmented by the addition of progesterone to estrogen treatment, providing further evidence that progesterone synergizes with estrogen in stimulating proliferation of the mammary tissue of the guinea pig. In this regard, the mammary glands of the guinea pig respond like those of the mouse (5), rat (8, 28) and rabbit (6, 7, 29). The concept of the two hormones acting concomitantly during pregnancy to elicit growth of the mammary alveoli is thereby given further experimental support.

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EVIDENCE FOR STIMULATION OF INSULIN SECRETION BY GROWTH HORMONE IN THE RAT[1]

FRANK L. ENGEL, THOMAS ALBERTSON,[2]
JOAN FREDERICKS AND ENRIQUE LOPEZ[3]

Departments of Medicine and Physiology, Division of Endocrinology, Duke University Medical Center, Durham, North Carolina

ABSTRACT

Based on the observation that only insulin will increase the concentration of glycogen in rat adipose tissue both in the presence and the absence of the pancreas, evidence for insulin secretion in response to growth hormone treatment was sought by measuring adipose tissue glycogen levels in fed intact and eviscerated rats. It had previously been shown that growth hormone had no influence on adipose tissue glycogen in 48-hour fasted rats, but the pancreas of these animals may be presumed to have been depleted of insulin. In fed rats given 2 ml. of 50 per cent glucose by stomach tube both insulin and growth hormone brought about significant increases in the glycogen content of the dorsal interscapular brown fat pad. Acutely eviscerated rats, which lack the pancreas, showed no accumulation of adipose tissue glycogen in response to a subcutaneous glucose load compared to intact controls unless given insulin. In the latter instance adipose tissue glycogen levels were comparable to those in intact rats given the same dose of glucose and insulin. Growth hormone had no influence on adipose tissue glycogen values in eviscerated rats even when given together with insulin. These data suggest that the pancreas is necessary for the adipose tissue glycogen response to growth hormone and that the effect is not mediated by growth hormone potentiating the action of circulating or tissue bound insulin. It is concluded that growth hormone stimulates the secretion or release of insulin from the pancreas, but data are reviewed which indicate that this is only one of several mechanisms involved in the action of growth hormone on carbohydrate metabolism.

THE mechanism of the hypoglycemic response to growth hormone in the rat has so far eluded adequate explanation. The fall in blood sugar is readily demonstrated in the fasted normal, adrenalectomized or hypophysectomized rat, (1, 2, 3) but not in the alloxan diabetic rat (1) except when the diabetes is mild, as in the experiments of DeGroot and Weber (4). There is one report of improved glucose tolerance following administration of growth hormone (5). Stimulation of insulin secretion or release of preformed insulin from the pancreas is suggested by these data.

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[2] Lederle Student Fellow.

[3] Eli Lilly Foreign Educational Fellow.

However, Anderson and Long were unable to obtain evidence for insulin secretion from a perfused pancreas preparation in vitro (6) and Randle failed to detect insulin activity in the plasma of growth hormone treated rats (7, 8). Moreover, growth hormone has been found to accentuate the fall in blood sugar when injected intravenously into eviscerated-hypophysectomized rats, animals which no longer possess pancreatic tissue (3). And finally, growth hormone has been shown repeatedly to have an insulin-like action *in vitro* on rat diaphragm (3, 7, 8, 9, 10) and on mammary gland slices from lactating rats (11). This action appears to depend on the presence of insulin in the tissue, since it cannot be demonstrated when tissues from diabetic animals are used, unless insulin is added (8, 10, 12).

In contrast to the rat, the evidence is stronger that insulin secretion or release is in fact provoked by growth hormone in other species. Thus, Bennett has adduced evidence for this in the dog using the pancreatic transplant technique (13), and Randle has detected an increase in plasma insulin activity in cats receiving growth hormone (8) and in humans suffering with acromegaly (14). On the other hand, it has been shown by Kurtz *et al.* (15) that in the dog growth hormone will still lower the blood sugar immediately after pancreatectomy, and Sirek and Best (16) found that growth hormone would depress the blood sugar in totally depancreatized dogs up to 24–72 hours after withdrawal of insulin. Thus insulin secretion cannot be the sole basis for the lowering of the blood sugar by growth hormone.

In 1950 we attempted unsuccessfully to obtain evidence for stimulation of insulin secretion by growth hormone in the rat by taking advantage of the observation that insulin seems to have the unique property of increasing the concentration of glycogen in adipose tissue (17, 18, 19). Using 48 hour fasted intact rats, with and without a glucose load, we were unable to elicit any increase in adipose tissue glycogen in growth hormone treated rats, even though hypoglycemia was induced (20). We concluded from these observations that the hypoglycemic response to growth hormone was not mediated by insulin secretion. Recently we have applied this technique to the analysis of the mechanism of action of the oral hypoglycemic agent, tolbutamide (21). During the course of these studies it became apparent that the fasted rat was a poor choice for demonstrating insulin secretion, since the pancreas of such an animal might be expected to be largely depleted of insulin and a peripheral action of insulin might be difficult to demonstrate. Thus, it was found that whereas tolbutamide had no influence on adipose tissue glycogen in the fasted rat given a glucose load, it brought forth a marked increase in glycogen in this tissue in the fed rat. This prompted us to reinvestigate the action of growth hormone in fed rats, with the finding that a prompt and striking increase in the glycogen content of adipose tissue could be induced. In the eviscerated rat, lacking the pancreas, no change in adipose tissue glycogen was to be seen.

METHODS

Male albino rats of the Vanderbilt strain, weighing between 170 and 230 grams were used. They were permitted free access to Purina rat chow and to water until the beginning of each experiment. Blood glucose was determined according to Somogyi (22) and liver and adipose tissue glycogen by the anthrone method of Carroll, Longley, and Roe (23) after precipitation of the glycogen from hot KOH with alcohol. Dorsal interscapular brown fat was dissected out and weighed on a Roller-Smith torsion balance. Adipose tissue glycogen was precipitated and prepared for analysis as previously described (19). Pentobarbital anesthesia was used only for removal of the liver and adipose tissue samples and for the evisceration procedure, which was done according to the technique of Russell (24). In the latter studies, the rats were kept in a chick-incubator set at 33° C to maintain body temperature postoperatively.

RESULTS

In Table 1 are found comparative values for blood glucose and liver and adipose tissue glycogen in 3 groups of fed rats, all of which had received 2 ml. of 50 per cent glucose by stomach tube at the beginning of the experiment and were sacrificed at the end of 3 hours. Group 1 was the control. Group 2 received 0.5 U. of glucagon-free insulin subcutaneously at the outset and Group 3 have been injected intraperitoneally with 4 mg. of bovine growth hormone (Somar-A Lot R50109 prepared by the Armour Laboratories for the Endocrinology Study Section of the National Institutes of Health). Reference to Table 1 reveals that growth hormone had no detectable influence on glucose tolerance, while insulin had the expected effect of lowering the blood sugar even in the face of the glucose load. The adipose tissue showed a marked increase in glycogen content in both the insulin and growth hormone groups as compared to the controls. There was no significant difference between the response to 0.5 U. of insulin and 4 mg. of growth hormone with respect to adipose tissue glycogen even though only the insulin induced a frank change in the blood sugar. Liver glycogen values were roughly comparable in the three groups, although somewhat lower in the growth hormone series.

In order to eliminate the possibility that growth hormone either had a direct effect on adipose tissue glycogen or potentiated the effect of circulating insulin without directly stimulating insulin secretion, an additional study was done using acutely eviscerated rats. In this experiment 800 mg.

TABLE 1. GLUCOSE TOLERANCE AND LIVER AND ADIPOSE TISSUE GLYCOGEN IN INTACT RATS

Experimental group	Blood glucose, mg. per 100 ml.				Glycogen, gm. per 100 gms.	
	0	1 hr.	2 hrs.	3 hrs.	Liver	Adipose Tissue
1. Control—2 ml. 50% Glucose p.o. (15)	115±2.0	143±3.8	148±3.4	134±3.2	4.42±0.312	0.277±0.054
2. 0.5 U. Insulin s.q.—Glucose (10)	120±4.0	85±3.6**	91±6.8**	118±8.6	4.00±0.170	0.820±0.127**
3. Growth Hormone 4 mg. i.p.—Glucose (16)	114±1.8	134±2.0	144±3.1	127±2.5	3.31±0.317	0.733±0.079**

Mean ± S.E.
** $P < .01$ compared to Control.

of glucose were administered subcutaneously and growth hormone, dissolved in physiologic saline, was slowly injected intravenously. The results are recorded in Table 2. Control rats exhibited markedly diabetic glucose tolerance curves and the adipose glycogen level was much lower than that recorded in the intact rats. Insulin in two doses, 0.05 and 0.5 unit each, brought about the expected improvement in glucose tolerance and an increase in adipose tissue glycogen. It is noteworthy that the mean glycogen level in the eviscerate rats receiving 0.5 unit of insulin and 800 mg. of glucose was identical with that in the intact rats receiving the same dose of insulin and 1000 mg. of glucose (Table 1). Eviscerated rats to which growth hormone had been administered exhibited blood sugar and adipose tissue glycogen values which were indistinguishable from those of the untreated eviscerated controls. From this experiment it would appear that a source of insulin is necessary in order for growth hormone to promote glycogen accumulation in adipose tissue. In order to determine whether this requirement was related to secretion or release of insulin from the pancreas or depended merely on the presence of adequate insulin in the organism, a final experiment was performed in which eviscerated rats received both 0.05 U. of insulin and growth hormone. The data, recorded in the last line of Table 2, fail to reveal any potentiation by growth hormone of the insulin effect on adipose tissue glycogen, or for that matter on the blood sugar.

DISCUSSION

These results lend strong but indirect support for the concept that growth hormone does indeed bring about a stimulation of insulin secretion in the rat. This interpretation is based on the observation made by several investigators that only insulin will increase the glycogen content of adipose tissue both in the presence and absence of the pancreas (17, 18, 19, 25). A glucose load, which is known to stimulate insulin secretion (26) likewise leads to accretion of glycogen in adipose tissue, provided that the pancreas is present. In the present experiments growth hormone had the same effect as insulin in intact fed rats, but not in eviscerated rats. Previously we had

TABLE 2. GLUCOSE TOLERANCE AND ADIPOSE TISSUE GLYCOGEN IN EVISCERATED RATS

Experimental group	Blood glucose, mg. per 100 ml.				Adipose tissue glycogen, gm./100 gm.
	0	1 hr.	2 hrs.	3 hrs.	
1. Control—8 ml. 10% Glucose s.q. (6)	120 ± 7.5	477 ± 33.8	548 ± 38.9	602 ± 26.8	0.051 ± 0.012
2. Insulin—0.05 U. and Glucose (7)	112 ± 2.3	409 ± 9.2	430 ± 34.3**	430 ± 41.6**	0.111 ± 0.023*
3. Insulin—0.5 U. and Glucose (5)	99 ± 5.2	231 ± 29.6**	150 ± 13.5**	108 ± 43.9**	0.916 ± 0.156**
4. Growth Hormone 4 mgm. i.p., Glucose (8)	109 ± 2.7	480 ± 15.4	566 ± 23.0	565 ± 19.9	0.035 ± 0.009
5. Growth Hormone, 4 mgm., Insulin, 0.05 U., Glucose (7)	98 ± 3.5	372 ± 16.2**	394 ± 23.1**	369 ± 34.0**	0.115 ± 0.034

Mean ± S.E.
** P <.01 compared to Control.
* P <.05 compared to Control.

found that it was ineffectual in the fasted rat (20). It seems reasonable to suggest that the basis for the difference in response of the fasted and fed rat lies in the low insulin content of the pancreas of the fasted animal (27). A similar difference was noted with tolbutamide, which likewise induced glycogen deposition in the adipose tissue of the fed but not the fasted rat (21). Unpublished studies demonstrate that tolbutamide also has no influence on adipose tissue glycogen in eviscerated rats. The failure of the eviscerated rat to respond to growth hormone even in the presence of injected insulin indicates that the increase of adipose tissue glycogen in the intact rats cannot be accounted for either by a direct action of growth hormone on adipose tissue or by a potentiation of the insulin effect.

Although these studies point clearly to a stimulation of insulin secretion, it is obvious from many different observations that all the immediate actions of growth hormone on carbohydrate metabolism are not so mediated. Indeed, there would appear to be at least three or four distinct mechanisms by which this hormone moderates carbohydrate metabolism. It influences glucose uptake and glycogen accumulation in diaphragm and skeletal muscle and this response seems to depend on the presence of insulin but is not mediated by insulin secretion. It brings about an increase in cardiac muscle glycogen which appears to be entirely independent of insulin (28). Thirdly, there is the response of adipose tissue glycogen which seems to be mediated directly by insulin secretion. And finally, it must be recalled that there are other metabolic changes which not only are not dependent on insulin, but actually represent contra-insulin responses. These include the diabetogenic effect, which is only partially accounted for by pancreatic exhaustion, the adipokinetic and ketogenic actions and the depression of the R.Q. (29, 30). Since the blood sugar response to growth hormone must represent the consequences of the interaction of these different effects it is apparent why changes in this parameter have been difficult to interpret. It explains too why it should be possible in the present experiments to observe an impressive insulin response in adipose tissue without seeing any change in the glucose tolerance in the same animal.

The demonstration that insulin secretion may occur in response to growth hormone administration is consistent with the evidence that insulin is necessary for the growth promoting effect of this hormone (31), but its significance for other metabolic parameters remains unclear. It should be emphasized that the present observations on adipose tissue are of limited physiological significance behond their use as an indicator of insulin secretion. The dorsal interscapular adipose tissue is made up largely of brown fat and represents only a very small proportion of the total adipose tissue. It was chosen because it is metabolically more active and responds more sensitively to insulin by glycogen accumulation than does white fat. No conclusions can be drawn from these experiments concerning the over-all role of growth hormone in adipose tissue metabolism in general.

The property of inducing hypoglycemia is not peculiar to growth hormone alone. It has also been demonstrated with prolactin (32, 33), ACTH (30, 34, 35) and thyrotropin (36). Foa *et al.* (32, 33) have presented evidence that insulin secretion is involved in the hypoglycemic response to prolactin in the dog. ACTH has an effect on adipose tissue glycogen in the rat which is quite comparable to that of growth hormone. This action can be demonstrated in the adrenalectomized animal and our data indicate that it is not due to contamination of the ACTH with other hormones. (To be published.) It thus represents another example of an extra-adrenal metabolic action of ACTH (37). There is no information available concerning the mechanism of the hypoglycemic action of TSH.

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THE CYCLICAL CHANGES IN THE MUCOSA OF THE GUINEA PIG'S UTERUS, CERVIX AND VAGINA AND IN THE SEXUAL SKIN, INVESTIGATED BY THE ELECTRON MICROSCOPE[1]

MARIO H. BURGOS[2] AND GEORGE B. WISLOCKI

Departments of Anatomy, Harvard Medical School, Boston, Massachusetts, and Cornell University Medical College, New York, New York

ABSTRACT

The mucosa of the guinea pig's endometrium, cervix and vagina and the epithelium of the perivaginal (sexual) skin have been studied with the electron microscope during estrus, metestrus and late diestrus.

During estrus, the uterine epithelial cells, the superficial mucous cells of the cervical epithelium as well as the basal and prickle cells of the vaginal epithelium display microvilli on their surfaces; these diasppear during diestrus.

Throughout the cycle in the cervix and during diestrus in the vagina, the superficial epithelial cells are filled with large droplets of a low-density material, apparently mucus. This seems to arise within distended vesicles of the endoplasmic reticulum in lower cells.

During estrus in the vagina and at all times in the sexual skin, the uppermost epithelial cells consist mainly of filaments. In the basal cells, these filaments occur in small clusters anchored on thickenings on the cell surfaces. Such cells contain an abundance of dense cytoplasmic particulates presumed to be ribonucleoprotein; an endoplasmic reticulum is absent or poorly developed.

The transitional layer of the vaginal epithelium during estrus shows a gradual retraction and apparent dissolution of the nuclei, paralleled by a swelling, vacuolization and disappearance of the mitochondria.

Some correlations have been drawn between the present observations and those reported earlier (*Endocrinology* **59**: 93. 1956) for the histochemical characteristics of these same tissues.

IN A previous paper (1), we reported the morphological and histochemical characteristics of the mucosa of the uterus, cervix, and vagina and of the epithelium of the sexual skin of the guinea pig in the four principal stages of the estrous cycle. This paper presents the results of parallel observations made on the fine structure of the same epithelia, as revealed by the electron microscope. Most of the reported observations refer to the two highly contrasting stages of the cycle, estrus and

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[2] Present Address: Instituto de Histología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina.

late diestrus, which reflect the influence of high and low levels of circulating estrogen, respectively (2).

MATERIAL AND METHODS

From many of the same guinea pigs used earlier (1), i.e., those in estrus, metestrus and late diestrus, we took 1 mm. strips of the uterus, the cervix, the vagina and the skin of the vaginal orifice. These were fixed for 3 hours in 1% buffered osmium tetroxide (pH 7.6), embedded in *n*-butyl methacrylate, sectioned transversely at about 25 mμ and examined either with an RCA EMU 2E or EMU 3B microscope (3). This procedure proved far more successful in preserving endometrium and vagina than cervix and sexual skin.

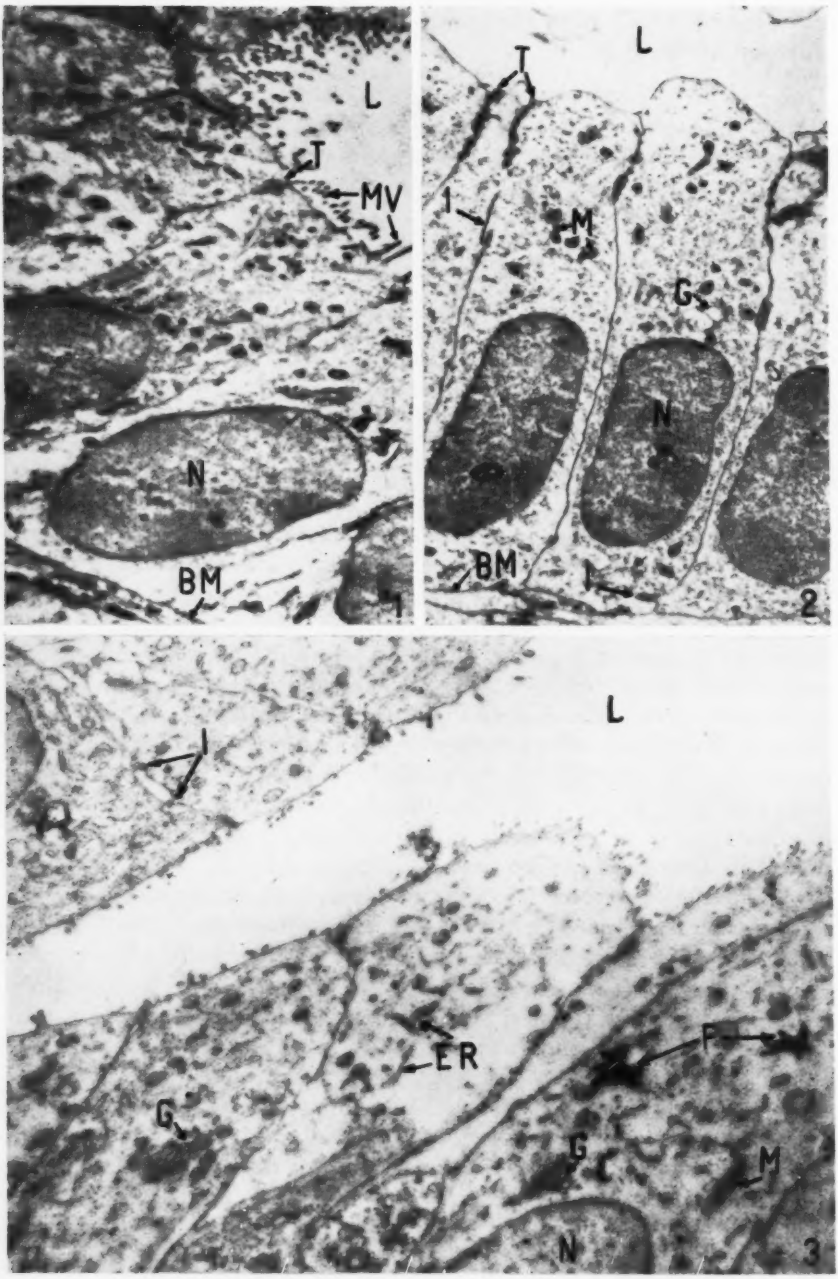
OBSERVATIONS

Endometrium

During *estrus*, the uterine epithelium both on the surface and in the glands consists of tall, columnar cells resting on a thin basement membrane (Fig. 1). The nucleus almost fills the lower half of the cell. The lateral membranes are relatively straight, exhibiting occasional folded interdigitations with adjacent cells. At the apical end, neighboring membranes thicken to form typical terminal bars; these are more conspicuous in the glands than on the surface epithelium (cf. Figs. 1 and 3). Short microvilli project from the apical surface of all the cells (Figs. 1 and 3). These vary in length and frequency, and some appear branched. The cytoplasm displays a variety of organelles: numerous dilated vesicles of endoplasmic reticulum containing a material of low electron density and studded with small dense granules 80–150 Å across, presumably ribonucleoprotein (4), small mitochondria containing relatively few internal cristae, supranuclear Golgi bodies (5), and, especially in the glandular cells, strands of delicate cytoplasmic fibrils. Lipide bodies, commonly distorted by the preparatory procedure, occur in many cells. Sometimes the lipides exhibit a complex nature, as described for other tissues (6, 7).

In *metestrus*, the endometrial cells, especially those on the surface, appear shorter than in estrus. The apical surfaces no longer show clearly-defined membranes and microvilli; irregular processes bulge into the lumen (Fig. 4). The terminal bars are far more conspicuous than earlier. The gland lumens contain some extremely dense masses as well as numerous irregular vacuoles, whose contents have been preserved poorly. These vacuoles appear continuous with saccular dilatations in the cell cytoplasm. The lipide inclusions are now much smaller and are typically rounded in shape.

During *diestrus*, the cells are strikingly smaller (cf. Figs. 1 and 2). This atrophy is reflected in a corresponding shrinkage of the nucleus and mitochondria. The outer membrane is now smooth. The cytoplasm contains only small vesicles of endoplasmic reticulum. The granular component appears relatively more abundant than at the other stages. The details of the lateral and basal membranes are clearer than at previous stages because of the relative absence of cytoplasmic inclusions.



(Explanation and key to abbreviations on facing page)

Cervical Epithelium

The cervix proper possesses two types of epithelial cells, one or two rows of basal cells and a row of superficial mucous cells (1). At all stages of the cycle, the basal cells resemble closely their counterparts in the diestrous vagina (Fig. 15). The overlying mucous cells are strikingly different from the basal cells. Their cytoplasm is filled with large saccular spaces containing a flocculent material of low density (Fig. 5). Interspersed are small mitochondria and small vesicles and tubules of endoplasmic reticulum, possessing clearly-defined membranes and surrounding dense granules. In estrus and metestrus, the outer surface of these cells is characterized by numerous microvilli (Fig. 5); during diestrus the surface is merely irregular. As in the vagina, leukocytes appear in the intercellular spaces of the mucosa during late metestrus and diestrus.

Vaginal Epithelium

In *estrus* the stratified epithelium, as seen with the light microscope, consists of basal germinative cells, a prickle-cell layer, a transitional layer in which the nuclei become flattened and pyknotic, and a thick cornified layer in which nuclei are rare. The electron microscope reveals considerably more detail about these various cell types, as represented schematically in Figure 6a.

The germinative cells send basal projections into an undulated basement membrane (Fig. 8). The inner aspect of this basal cell membrane shows dense thickenings, which fuse with packets of 150 Å-thick cytoplasmic fibrils, as has been described for skin (8). All other surfaces of the germinative cells are quite irregular (Fig. 7) and resemble the surfaces of the over-

Key to abbreviations on figures

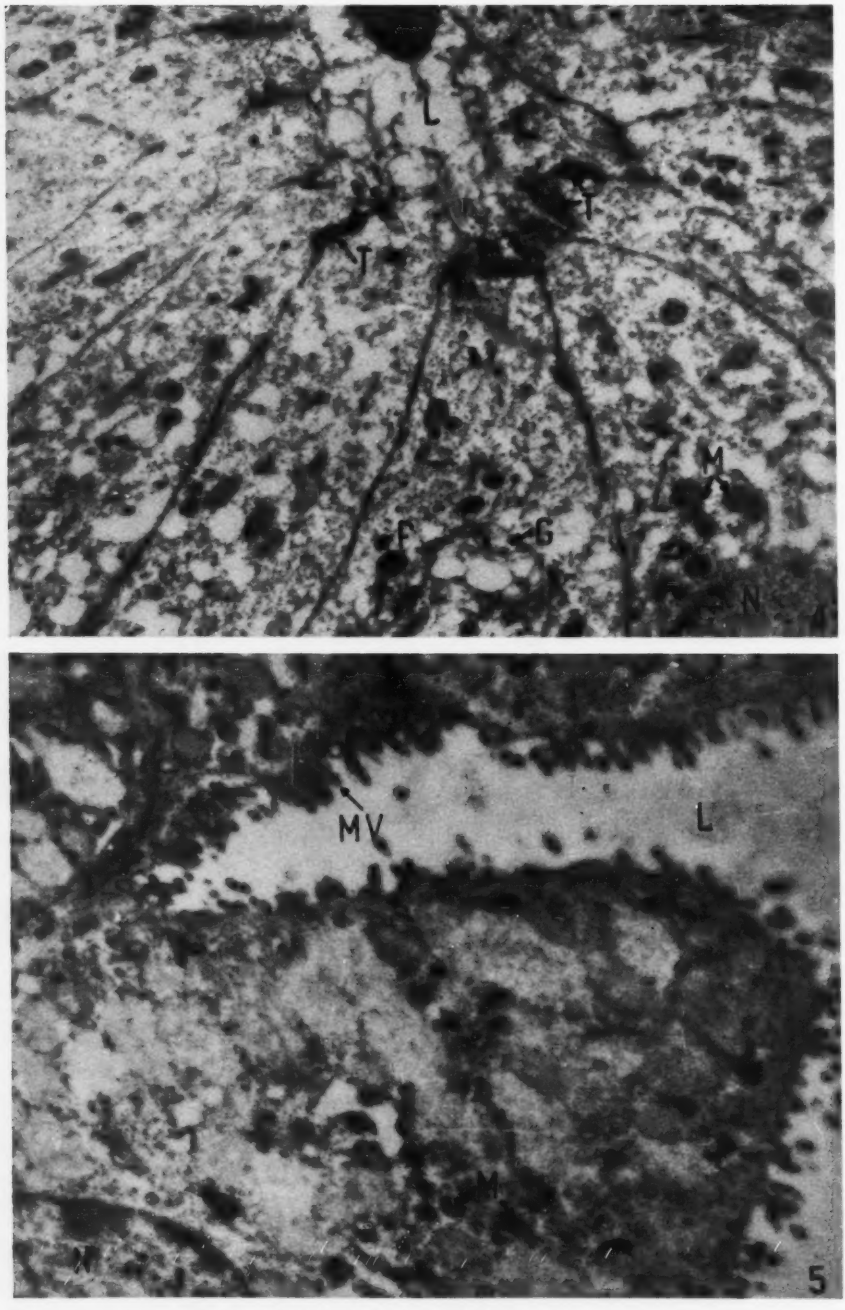
BM, basement membrane	I, interdigitation
BV, blood vessel	L, lumen
C, collagen fiber	M, mitochondrion
CF, clusters of filaments	MV, microvillus
D, desmosome	N, nucleus
E, endothelium	P, particle
ER, endoplasmic reticulum	PS, perinuclear space
F, lipide droplet	S, intercellular space
G, Golgi complex	T, terminal bar

FIGS. 1–3. ELECTRON MICROGRAPHS OF THE UTERINE EPITHELIUM.

FIG. 1. A portion of a uterine gland during estrus, illustrating the microvilli at the surface of the cells. ×4,400.

FIG. 2. The smooth surface, without microvilli, of a uterine gland segment during diestrus. Compare the sizes of the cells with those of Figure 1, which was taken at a lower magnification. ×8,300.

FIG. 3. A part of the surface epithelium and the pit of a gland during estrus, revealing the small size of the microvilli on the surface cells. ×15,000.



(See facing page for legends)

lying prickle cells. Adjacent cells appear joined together by periodic thickenings of their membranes, a pair of such thickenings corresponding to the desmosome of light microscopy. A half-desmosome resembles the basal densities described above and likewise serves as an anchor for a cluster of cytoplasmic filaments. Only a thin space separates the half-desmosomes. Between two desmosomes, the intercellular space is greatly distended, and each cell membrane sends out periodic groups of microvilli. In some locations, the microvilli of the two adjacent cells interdigitate (Fig. 7); in other places, one cell possesses the microvilli and the opposite cell exhibits a concavity (Fig. 11).

Internally, as on their surfaces, the basal cells and prickle cells are essentially alike. The nuclei are large and somewhat lobulated. They possess a well-defined nucleolus, typical conglomerations of light granules (chromatin), and numerous, isolated, dark 80–150 Å granules. The cytoplasm is crowded with similar particles (Figs. 11; 12 a, b). An endoplasmic reticulum is poorly distinguishable. Mitochondria exhibiting a dense matrix and few internal cristae are randomly distributed. Clusters of cytoplasmic fibrils lie in the peripheral cytoplasm. Such clusters appear to be more abundant in the upper layers of prickle cells than in the underlying ones.

In the transitional layer, desmosomes are not apparent, and microvilli become irregular and infrequent (Fig. 10). The cells are flattened. Dense bundles of filaments crowd the cytoplasm. In the lower cells of this layer, the nucleus is highly irregular in shape and appears pale because it contains fewer dense particles than do the underlying layers and because the small, light components have accumulated near the nuclear membrane (Fig. 12 c, d). In the outermost cells of the layer, the nucleus is reduced to a dark strand (Fig. 10). A clear lacunar space surrounds the nucleus in cells lying in the outer portion of the transitional layer (Fig. 12 d, e), apparently as a result of shrinkage of the nucleus proper away from its outer envelope (9). The mitochondria undergo pronounced swelling in the transitional layer (Fig. 12c), become conspicuously vacuolated in the outer cells and eventually disintegrate (Fig. 10).

The outermost, fully cornified cells are flattened, with serrated surfaces and thick outer membranes (Fig. 9). Nuclei and organelles are no longer distinguishable. The cells contain one or more large clear spaces surrounded by dense bundles of filaments.

FIG. 4. Electron micrograph of a part of the epithelium of a uterine gland during metestrus. Irregular processes and clear vacuoles bulge into the lumen, which is partially occupied by a large, extremely dense mass. Cytoplasm is filled with saccular dilatations, small vesicles and dense particles. ×7,100.

FIG. 5. Electron micrograph of a portion of the cytoplasm of two mucous cells of the uterine cervix during estrus. Numerous microvilli project into the lumen of the cervical canal. Large globules with poorly-defined limits, containing a flocculent material of low-density mucus, fill up the cytoplasm. ×13,500.

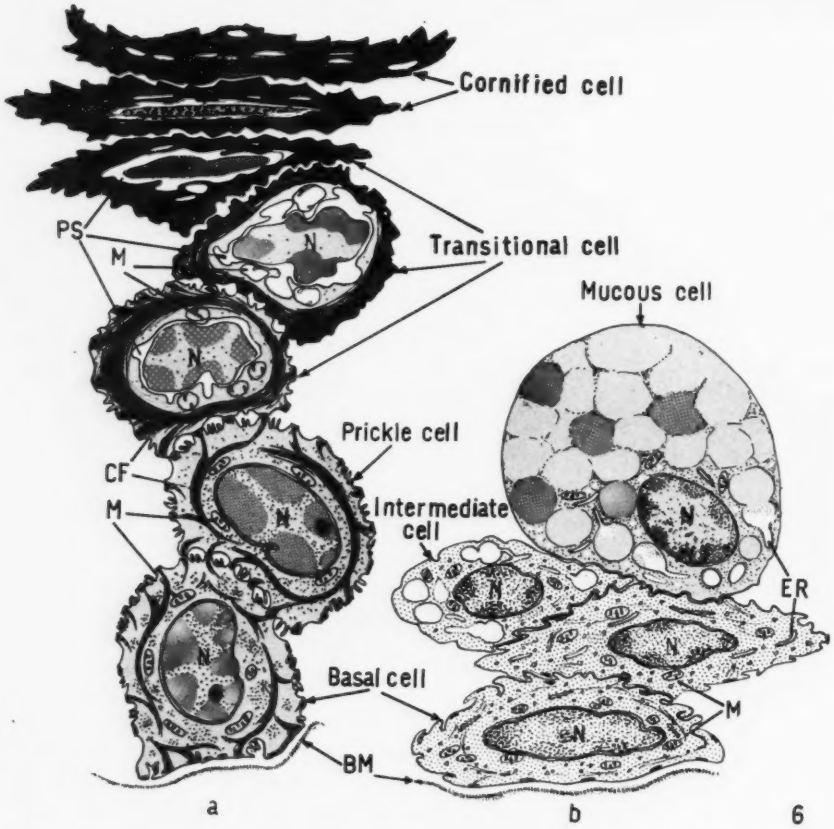
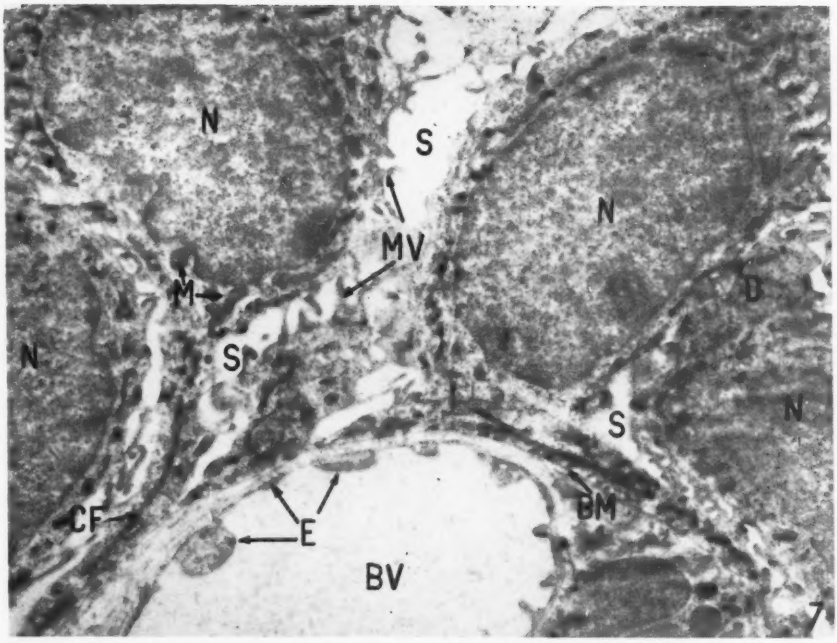
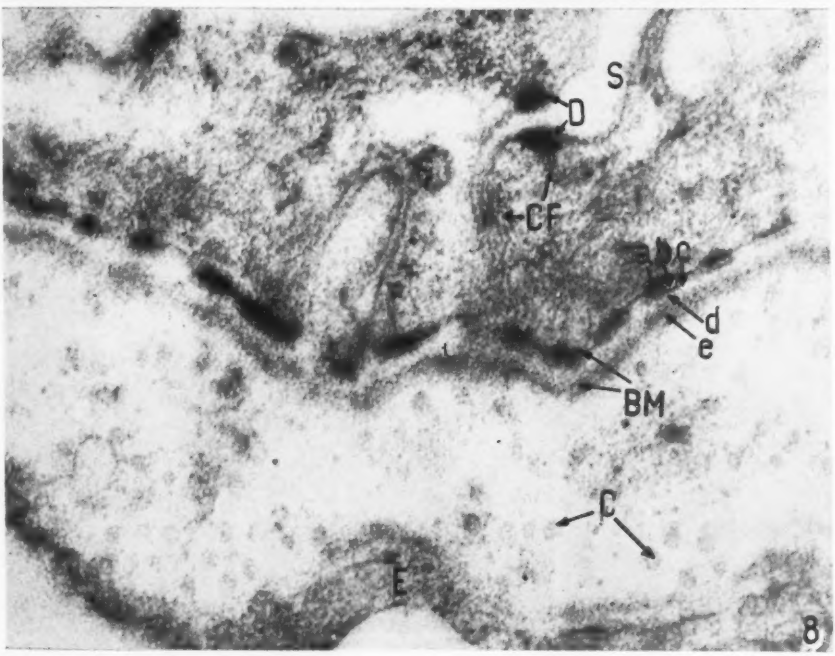


FIG. 6. Diagrammatic sections through the vaginal epithelium at two different stages of the estrous cycle.

a. During estrus, intercellular microvilli and channels appear in the basal and prickle-cell layers; they gradually disappear at the transitional layer and are completely absent from the cornified cells. The nuclei present striking changes at the transitional-cell level: diminution of the dark, granular component (RNA), shrinkage away from the outer membrane to produce a perinuclear space, and concentration of the nuclear contents in a dense strand. In the cytoplasm, there is an increase in the number of filaments from the basal to the outer layers, and a reduction in the number of dark particles (RNA). Mitochondria swell at the transitional-cell level and disintegrate in the cornified layer. Apparently they also contribute to the clear spaces around the shrunken nuclei. The serrated scales of the outer layer present an extremely dense cytoplasm filled with closely-packed clusters of filaments and clear spaces without membranes. Nuclear remnants are no longer distinguishable from the dense background.

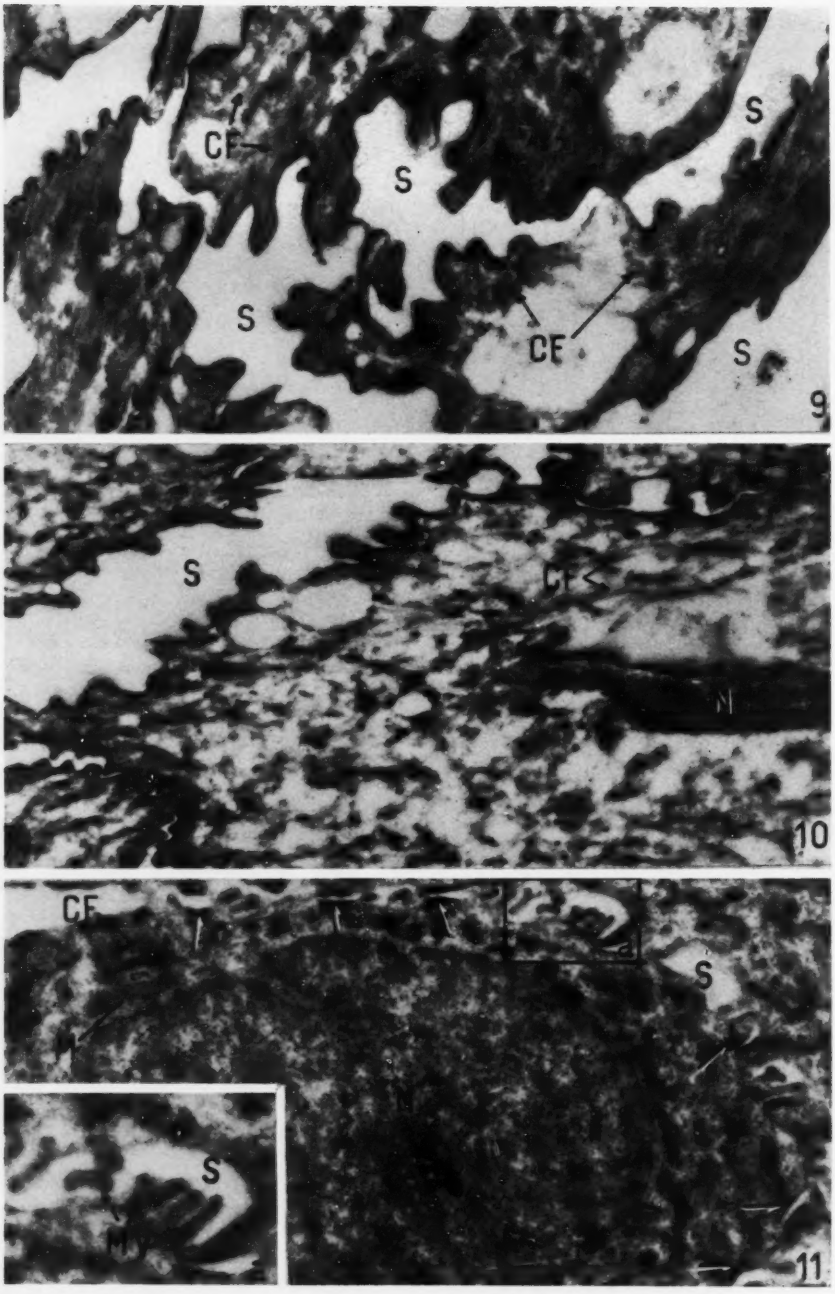
b. During late diestrus the vaginal epithelium has 3 layers of cells: in these relatively narrow limits the complete process of mucification takes place. Endoplasmic reticulum develops into vesicles, tubules, channels and cisternae. A low-density material, flocculent and amorphous, accumulates inside these saccular dilatations of the endoplasmic reticulum; almost abruptly the outermost cell is transformed into a typical mucous cell. The diagram illustrates the communication of the large droplets of mucus with the endoplasmic reticulum. Intercellular spaces are narrow and without microvilli; the cells frequently interdigitate.





FIGS. 7–8. ELECTRON MICROGRAPHS OF THE VAGINAL EPITHELIUM DURING ESTRUS.

(*See page 115 for explanation of figures*)



FIGS. 9, 10 AND 11. ELECTRON MICROGRAPHS OF CELLS OF THE VAGINAL EPITHELIUM DURING ESTRUS. (All ×25,000.)

(*Explanation of figures on facing page*)

During *metestrus*, the same layers appear. Microvilli have become less numerous on the cell surfaces of germinative and prickle cells. Ribonucleoprotein granules crowd the cytoplasm of these lower cells. Bundles of thick filaments are far more abundant in the basal, prickle-cell and transitional layers than during estrus.

By *late diestrus* the vaginal epithelium is reduced to three layers of cells: a basal or germinative layer, an intermediate layer and an outer mucous layer (Fig. 6b). Occasional leukocytes appear within the vaginal epithelium in distended intercellular spaces.

The basal cells differ from their appearance in estrus in the following respects. They are much smaller and distinctly flattened (Fig. 15). The flattened nucleus generally lacks a nucleolus, and conglomerations of light granules (chromatin) lie against the nuclear membrane. The intercellular spaces are not distended, so that the lateral membranes of adjacent cells are closely apposed and possess no microvilli. These surfaces interdigitate extensively, however (Fig. 14). Desmosomes appear reduced in number. The bundles of cytoplasmic filaments and the free ribonucleoprotein particles are likewise much less numerous. On the other hand, collapsed vesicles of endoplasmic reticulum, indistinguishable in the preparations

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EXPLANATION OF FIGURES 7–11

FIG. 7. The basal germinative cells send basal projections into the basement membrane. Clusters of filaments cross the cytoplasm in different directions and fuse with the desmosomes. Groups of microvilli project from the cell surfaces into the intercellular spaces. Below the basement membrane, a portion of a blood capillary is seen with a rather curious disposition of the endothelium in thick and thin portions. ×10,500.

FIG. 8. A higher magnification of the basement membrane to show: *a*, dense thickenings in the inner aspect of the basal cell membrane, *b*, a clear narrow line, *c*, the outer aspect of the cell membrane, *d*, a clear space containing a material of low density, and finally *e*, a denser band in contact with the connective tissue. Cross sections of collagen fibers and capillary endothelium are seen in the connective tissue of the lamina propria. Note, in the cytoplasm of the basal cells, clusters of filaments anchoring in desmosomes. ×85,000.

FIG. 9. Fully cornified cells of the outer layer with serrated surfaces and thick cellular membranes. Nuclei and organelles are no longer distinguishable. The cells contain one or more large clear spaces surrounded by dense bundles of filaments. Between the scales the intercellular spaces appear extremely irregular and wide.

FIG. 10. Cell from the outermost transitional layer. The thick and irregular surface of the cell receives dense bundles of filaments, but desmosomes are not apparent. The nucleus is reduced to a dense strand surrounded by irregular, clear spaces. No cytoplasmic organelles are recognizable.

FIG. 11. Prickle cell. The cytoplasm is crowded with small particles. Mitochondria and clusters of filaments surround the nucleus. Arrows point to desmosomes at the cell surfaces; between desmosomes the intercellular spaces are greatly distended and each cell membrane sends out periodic groups of microvilli. *Inset a.* A portion of the cell surface, magnified 60,000×, showing a group of microvilli projecting into the intercellular space.

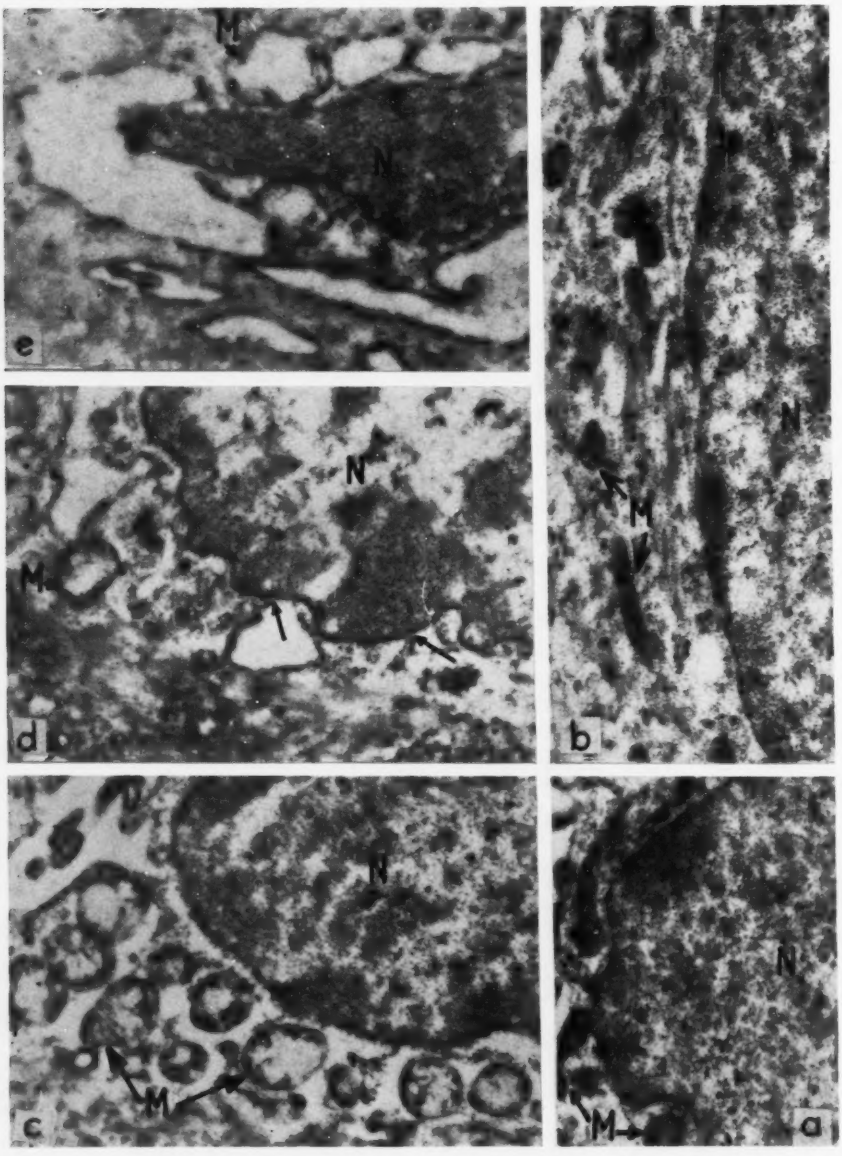


FIG. 12. ELECTRON MICROGRAPHS OF VAGINAL EPITHELIAL CELLS DURING ESTRUS. (All ×25,000.)

a. Basal cell. The nucleus contains numerous dark and light particles. The mitochondria have a dense matrix and few cristae. Cytoplasmic dark particles are interspersed between the mitochondria.

b. Prickle cell. Numerous dark particles in the nucleus and in the cytoplasm. The

from estrus, are now apparent and show the characteristic studding of ribonucleoprotein particles.

In the overlying cells, which correspond to the prickle cells of estrus, intercellular spaces are likewise poorly developed. The elements of endoplasmic reticulum are distended into cisternae and lacunae, containing material of low electron density (Fig. 13).

The outermost mucous cells contain a central nucleus, an intermediate zone filled with large, pale, flocculent droplets having poorly-defined membranes, interspersed with mitochondria, and a thin outer layer of cytoplasm containing thin canalicular elements of endoplasmic reticulum surrounded by dense particles (Fig. 15). The luminal surface is smooth.

Epithelium of the Sexual Skin

Three strata can be distinguished in the epithelium of the vaginal vestibule: a basal layer occupying about one-half of its thickness, a granular layer and a keratinized layer.

No changes are conspicuous in the basal layer between *estrus* and *diestrus* in our preparations. The nuclei are large, with quite regular surfaces and prominent nucleoli of reticular structure. The cells are voluminous and possess smooth contours. The cell membranes display periodic dark thickenings or desmosomes; the cells resting on the basement membrane show corresponding thickenings on the inner aspect of the basal membrane (see vagina). The cytoplasm contains sparse bundles of thin filaments, which cross the cell and anchor on the surface thickenings. Mitochondria are numerous and extremely large, with a pale matrix and abundant internal cristae. Endoplasmic reticulum is inconspicuous, but the cytoplasm is peppered with ribonucleoprotein particles. Scattered in the subnuclear cytoplasm are large, irregularly ovoid, extremely opaque bodies (melanin (1)), with no apparent internal structure. Occasionally these bodies are surrounded by smaller particles of similar density.

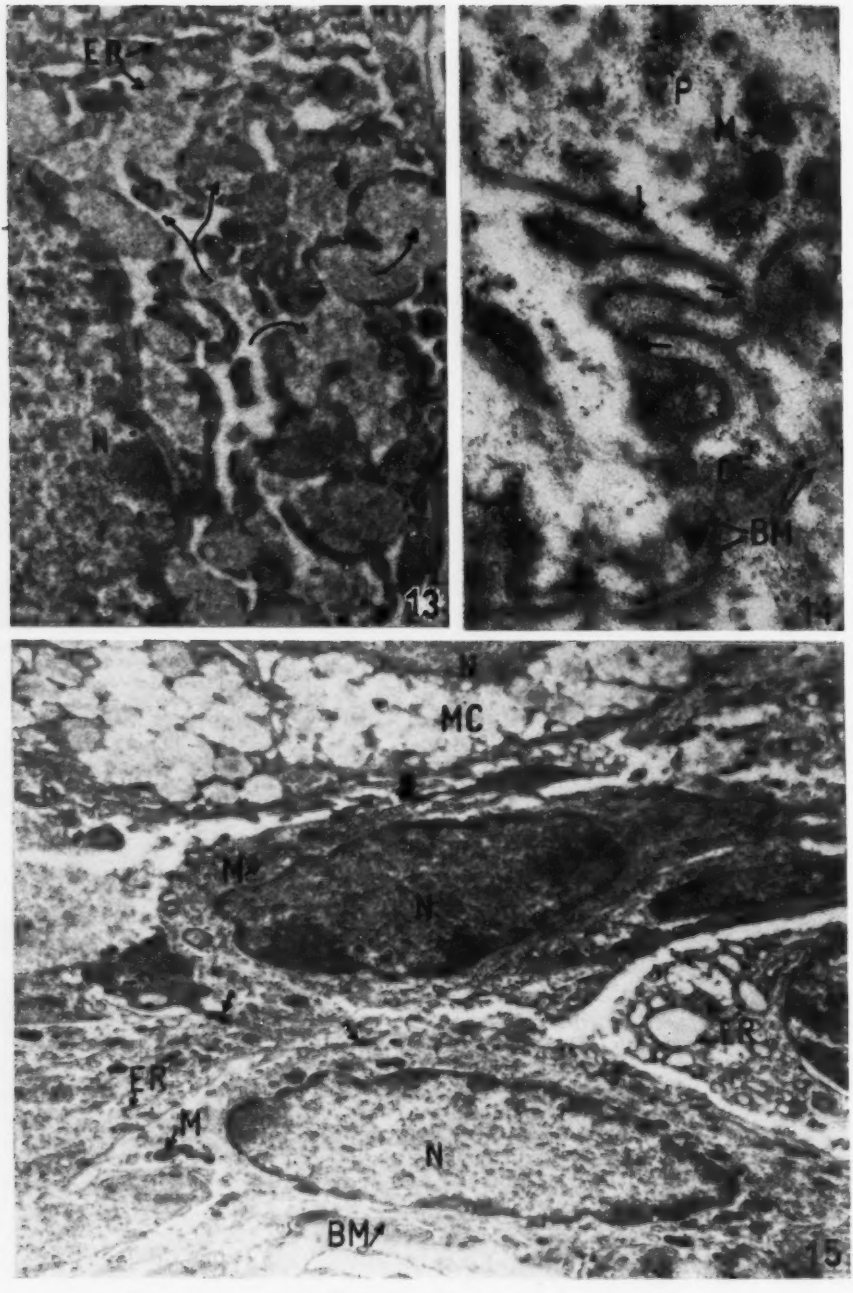
In the cells of the overlying stratum granulosum, the nucleus is somewhat smaller. The cells remain smooth in contour, with periodic desmosomes. The mitochondria appear smaller and denser than in the underlying cells. The cytoplasm displays abundant dense bundles of filaments. Three types of granules, all possessing the same density, occur in the cytoplasm: small particles 80–150 Å in diameter, larger round bodies typi-

mitochondria are similar to those of the basal cells.

c. Transitional cell (lower part of layer). The nucleus appears to have fewer dark particles than do the basal and prickle-cell nuclei. The mitochondria are swollen and possess a low-density matrix.

d. Cell from the middle portion of the transitional layer. The nucleus is shrunken away from its outer membrane, leaving clear spaces indicated by the arrows. There are fewer dark particles, and the mitochondria are pale and swollen.

e. Cell from the outer part of the transitional layer. Intense shrinkage of the nucleus. The perinuclear space is more prominent, and the mitochondria are extremely swollen.



FIGS. 13, 14 AND 15. ELECTRON MICROGRAPHS OF THE VAGINAL EPITHELIUM DURING LATE DIESTRUS.

(*See explanation of figures on facing page*)

cally about 60 mμ across, and extremely large, irregular conglomerates generally about 0.5 μ in diameter. The two larger types are far less numerous in *diestrus* than in *estrus*.

The outermost keratinized layer appears abruptly, with no detectable transition. The cells consist of flattened, homogeneous scales without recognizable nuclei or nuclear spaces. From the base of this layer outward, the scales are at first dark, then light and finally extremely dark. This sequence reflects changing osmiophilia rather than changing density, as indicated by examination of such preparations under the light microscope.

DISCUSSION

Our discussion of these observations will concentrate on three major points: (a) the development of microvilli on many cells of the genital tract epithelia during estrus; (b) the differing cytological manifestations of mucification and cornification of the stratified epithelia, and (c) certain correlations that can be drawn between the present observations on ultrastructure and our earlier histochemical results on the histochemistry on the same tissues (1).

Microvilli. During estrus, there is a common morphological response on viable cells along the genital tract of the guinea pig, namely, the presence of microvilli. These occur on the free borders of the uterine epithelial cells and the cervical mucous cells, and on the surfaces of the lower cells in the vaginal epithelium. They do not develop in the sexual skin. These microvilli are relatively short and sparse, thus resembling those in gall bladder (7) and trachea (10). They are far less crowded and organized than in highly specialized surfaces like the brush border of the renal proximal tubule (11), the striated borders in the small intestine (12) or the testicular ductuli efferentes (13). Such an augmentation of the cell surface is probably associated with absorption. Mucous-secreting cells may require fluid to hydrate their product before its release. In the vagina, such a differentia-

FIG. 13. A portion of the cytoplasm and nucleus of a mucous cell, revealing the extraordinary dilatations of the elements of the endoplasmic reticulum into cisternae and lucunae, containing a pale, flocculent material (mucus). Arrows point to the communications of the distended endoplasmic reticulum. ×50,000.

FIG. 14. A portion of the cytoplasm of two basal cells and of the basement membrane. The intercellular space is narrow and lacks microvilli and dilatations. Cells interdigitate frequently (arrows). Present are mitochondria with dense matrix, conglomerations of dark particles, and poorly-defined clusters of filaments, which anchor in dense thickenings of the cytoplasmic membrane. A clear space followed by a dense line in contact with the connective tissue completes the basement membrane. ×75,000.

FIG. 15. Vaginal epithelium reduced to three layers of cells. At the bottom, a basal or germinative cell with a flat nucleus, scattered mitochondria, and canaliculi and vesicles of endoplasmic reticulum. Desmosomes (arrows) are less prominent than during estrus. In the middle, intermediate cells with distended endoplasmic reticulum containing low-density material (mucus). In the upper portion of the figure lies a mature mucous cell containing flocculent droplets in poorly-defined membranes. ×8,000.

tion may reflect the high requirement for nutrients by rapidly growing and differentiating cells.

Cornification and Mucification. The outermost cells of the vaginal stratified epithelium become cornified during estrus; those of the perivaginal epidermis are regularly keratinized. In both locations, the more basal cells are characterized by fairly numerous bundles of fine cytoplasmic filaments, 150 Å thick, and by an abundance of isolated or clustered dense particles of ribonucleoprotein (4, 14). Typical endoplasmic reticulum is not distinguishable (8). In the upper layers the fiber clusters become more abundant and fuse, while the granules decrease in number. It has long been postulated that ribonucleoproteins are somehow intimately concerned with protein synthesis (15), and the present association is compatible with this hypothesis.

There are, of course, differences in the cytomorphosis exhibited in the cornifying vaginal epithelium and in the keratinizing sexual skin. Peculiar to the vagina are signs of cell degeneration in the transitional layer. The nucleus shrinks away from its outer envelope, the nuclear contents eventually disintegrating. The surrounding mitochondria swell and finally disappear. In this connection, it may be pertinent that Dounce *et al.* (16) found that as mitochondria swell they apparently release an enzyme that detaches desoxyribonucleic acid from its protein.

Characteristic of the sexual skin is the development of large aggregates of what are apparently nucleoprotein bodies in the stratum granulosum; it is in this layer that clusters of cytoplasmic filaments first become very numerous. In the keratinized scales, the "cytoplasm" is almost homogenous, as though the filaments had become fused.

The alternative specialization observed here in stratified epithelia is mucification of the outermost layer. This condition obtains throughout the cycle in the cervix and during estrus in the vagina. The major observation of interest in this connection is the appearance of low-density material in the underlying cells. This is presumably mucus within vesicles of the endoplasmic reticulum. As usual, the vesicular system is surrounded by small dense particles (apparently ribonucleoprotein). Thus, mucus formation occurs not free in the cytoplasm but within pre-existing membranes. This pattern resembles that described by Palade (17) for the formation of prozymogen granules in the pancreatic acinar cells.

Histochemical correlates. In our earlier study (1), we noted that the cytoplasm was especially basophilic in the endometrial cells during late diestrus, in the basal cells of the vaginal epithelium during metestrus and diestrus, and in the stratum granulosum of the sexual skin (keratohyalin granules) during estrus. Electron microscopy reveals that at these times there is a particular abundance of the small dense particulates. Evidence has also been adduced that keratohyalin granules contain ribonucleoprotein (18).

In the vagina during estrus and in the stratified epithelium of the skin, there is a direct correlation between the degree of cytoplasmic acidophilia and the sulfhydryl-disulfide reaction and the abundance of clusters of cytoplasmic filaments.

A final point of interest is the high level of alkaline phosphatase and glucose-6-phosphatase in active cells bearing microvilli. These enzymes are abundant in many endometrial cells and in the basal layers of the vagina during estrus. Such enzymes appear in some instances to be concerned with transport across membranes, so that their abundance in cells possessing increased surfaces assumes added significance.

Acknowledgment

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MAIN EFFECTS AND INTERACTIONS OF CORTISONE, GROWTH HORMONE AND TRIIODOTHYRONINE IN PRODUCTION OF TEMPORARY DIABETES IN THE RAT[1]

JOHN A. OWEN, JR.[2] AND FRANK L. ENGEL

Departments of Medicine and Physiology, Division of Endocrinology, Duke University Medical Center, Durham, North Carolina

ABSTRACT

The diabetogenic effects of cortisone, triiodothyronine, growth hormone, and all possible combinations thereof were studied in the intact tube-fed rat. The design of the experiment was factorial, suitable for the analysis of variance. Growth hormone produced hyperglycemia, glycosuria, nitrogen retention and a gain in weight. The other hormones produced hyperglycemia and glycosuria with nitrogen wastage and weight loss. Every combination of hormones produced changes in each parameter equivalent to the additive effect of the individual hormones. No significant synergistic or antagonistic interactions were demonstrated.

SEVERAL workers have produced temporary diabetes in the intact rat with various single hormonal agents: growth hormone (1), adrenocortical hormone (2), and thyroid hormone (3). However, the knowledge gained from these studies does not permit prediction of the effects of various combinations of these diabetogenic hormones, and investigation of such effects has not produced conclusive results.

In this laboratory temporary diabetes has been produced regularly in the intact, tube-fed rat with a combination of adrenocortical hormone (ACTH, and later cortisone) and growth hormone (4, 5). Neither hormone alone was diabetogenic under the particular conditions of these studies, and we assumed that the diabetic response represented a synergism, based on the metabolic interactions of the two hormones in protein and carbohydrate metabolism. More recently we have found triiodothyronine also to be diabetogenic in the tube-fed rat, whether it be administered alone or in combination with either cortisone and growth hormone (6). However, the study as done did not permit definite conclusions as to the nature of the combined effects of the three hormones, since the only parameters studied

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[2] American Diabetes Association fellow. Present address: Department of Internal Medicine, Mt. Alto Veterans Administration Hospital, Washington, D. C.

were body weight, glycemia, and glycosuria, and the statistical method did not adequately distinguish between additive and synergistic effects.

For these reasons the study was expanded to include measurement of nitrogen balance and repeated using a factorial design suitable for the analysis of variance. As discussed by Fisher (7), this experimental design makes it possible to analyze variations within treatment groups as well as interactions between two or more treatments.

The present investigation, designed and analyzed in this fashion, shows that each of the three hormones is diabetogenic under the conditions of the experiment, and that in no case is there any significant interaction in any of the parameters, i.e., the combined effects are additive rather than synergistic.

METHODS AND MATERIALS

Male albino rats of the Vanderbilt strain, weighing 250–350 gm., were housed in

TABLE 1. DESIGN OF EXPERIMENTAL GROUPS

Treatment	Cortisone (mg.)	Growth hormone (mg./100 gm.)	Triiodothyronine (μg./100 gm.)
1	—*	—*	—*
2	2.5	—	—
3	—	2.0	—
4	2.5	2.0	—
5	—	—	5
6	2.5	—	5
7	—	2.0	5
8	2.5	2.0	5

* Denotes use of the appropriate control solution.

individual metabolism cages in an air-conditioned room maintained at 28°–30° C. They were tube-fed twice daily a high-carbohydrate liquid diet, containing 0.45 gm. of carbohydrate, 0.07 gm. of protein, 0.009 gm. of fat, and 2.21 calories per ml. For any given day, the total volume of tube-feeding was 11 ml. per 100 gm. of body weight as measured that morning.

Animals previously adapted to tube feeding were assigned at random to each of the 8 treatment schedules (Table 1). The experiment consisted of 9 series of 8 rats each, studied over a period of two control days followed by four treatment days.

Cortisone acetate was suspended (5 mg./ml.) in physiologic saline and injected subcutaneously in the left flank. Growth hormone (Horner's PR-1, from pork pituitary, prepared by the Raben-Westermeyer method) was dissolved (10 mg./ml.) in distilled water acidified to pH 4 with 0.2 N HCl; it was injected subcutaneously in the right flank. l-Triiodothyronine was dissolved (50 μg./ml.) in distilled water brought to pH 9.0 with 0.1 N NaOH; it was injected intraperitoneally. The control materials were: (1) for cortisone, physiologic saline; (2) for growth hormone, human serum albumen in aqueous solution acidified to pH 4.0; and (3) for triiodothyronine, distilled water brought to pH 9.0. In every case the volume, site, and route of injection for the control solutions were the same as for the appropriate hormone.

Daily weights (to the nearest gram) were recorded each morning, followed by the tube-feeding, and injection on days 3–6. Three hours after tube-feeding, 0.2 ml. of blood was obtained from the tail vein without anesthesia (days 1–2 and 5–6) and analyzed in

duplicate for glucose by the method of Somogyi (8). A complete urine and stool collection was made daily and pooled 48 hour urine specimens (days 1–2, 3–4, and 5–6) were analyzed for nitrogen by the micro-Kjehldahl method, and for sugar by the method of Benedict (9). Each new batch of diet was analyzed in triplicate for nitrogen concentration. Nitrogen content of the pooled 48 hour stool collections was estimated from their dry weight.[3]

Results are expressed as follows: (1) "net increase in blood sugar" refers to the mean blood sugar (in mg.%) for the last two days of treatment less the mean value for the two control days; (2) "net glycosuria" represents the mean 48-hour glucose excretion (in mg.) for the four treatment days less the value for the two control days; (3) "net nitrogen balance" is the mean net 48 hour balance (in mg. of nitrogen) for the four treatment days less the net balance for the two control days (net balance is defined as nitrogen intake less the sum of fecal and urinary nitrogen); and (4) "net change in weight" is the mean daily gain or loss in weight (in gm.) for the four days of treatment less the mean daily gain or loss for the two control days. In the statistical analysis, the values for net glycosuria (in mg.) are transformed to their logarithms.

These data are analyzed by application of the analysis of variance to a 2^3 factorial design (10). This method is described in some detail since it is not often used in biological research.

The results for any parameter are arranged in columns according to the treatments and in rows according to the replications. The following is an example:

Series	Treatment								Total
	Control	I	II	I+II	III	I+III	II+III	I+II +III	
1	a	b	c	d	e	f	g	h	Z′
2	i	j	k	l	m	n	o	p	Z
Total	Q	R	S	T	U	V	W	X	Y
Mean	q	r	s	t	u	v	w	x	

Definitions:

1. Adjustment for the mean $= \frac{Y^2}{16}$
2. Treatment sum of squares $= \frac{Q^2+R^2+S^2+T^2+U^2+V^2+W^2+X^2}{2} - \frac{Y^2}{16}$
3. Replications sum of squares $= \frac{(Z)^2+(Z')^2}{8} - \frac{Y^2}{16}$
4. Total sum of squares $= a^2+b^2+c^2 \cdots p^2 - \frac{Y^2}{16}$
5. Residual sum of squares = Total sum of squares − (treatment sum of squares + replications sum of squares).
6. Degrees of freedom:
   a. ascribable to treatments = 7
   b. ascribable to replications = 1
   c. ascribable to error = (16 − 1) − 7 − 1 = 7

[3] Previous studies have shown a significant correlation between dry stool weight and nitrogen content in experiments using this tube-feeding. The regression equation is N Content (mg.) = 45.17 + 24.24 × Stool weight (gm.). The correlation is not significantly altered by the various hormonal treatments. The fiducial limits, at the 5% level, are ±38 mg., a relatively minor range in view of the total nitrogen excretion of these animals.

7. Mean error variance $= \dfrac{\text{Residual sum of squares}}{7}$

8. Standard error of the mean $= \sqrt{\dfrac{\text{mean error variance}}{2^{n-2}\,r}}$ where n = number of treatments and r = number of replications; here $2^{n-2}r = 2^{3-2} \times 2 = 2 \times 2 = 4$

9. Main effect of treatment I $= \frac{1}{4}(x+v+t+r) - \frac{1}{4}(w+u+s+q)$
10. Main effect of treatment II $= \frac{1}{4}(x+w+t+s) - \frac{1}{4}(r+u+v+q)$
11. Main effect of treatment III $= \frac{1}{4}(x+w+v+u) - \frac{1}{4}(t+s+r+q)$
12. Interaction of I+II $= \frac{1}{4}(x+t+u+q) - \frac{1}{4}(v+w+r+s)$
13. Interaction of I+III $= \frac{1}{4}(x+v+s+q) - \frac{1}{4}(t+w+r+u)$
14. Interaction of II+III $= \frac{1}{4}(x+w+r+q) - \frac{1}{4}(t+v+s+u)$
15. Interaction of I+II+III $= \frac{1}{4}(x+r+s+u) - \frac{1}{4}(t+v+w+q)$

The standard error is divided into each main effect and each interaction to give a value for t; using the number of degrees of freedom ascribable to error (in this case 7), the P value for each t may be found in the standard tables of t.

In several of the series, an occasional animal died before the study period was over, necessitating the inclusion of an extra animal in the next series. Because of this mingling of the series, an analysis of the variance arising from differences among the various replications was impossible. In view of the relative agreement among the replications this does not invalidate an analysis of the differences among treatments. The only practical result of this omission is that the error variance is actually measuring to a certain extent the differences among replications as well as the residual error. It can be shown that in this case the contribution of the former is negligible.

In the tables that follow the degrees of freedom ascribable to treatments is always 7 and hence the degrees of freedom for error is always 64. The term $2^{n-2}r$ works out to 18 ($2^{3-2} \times 9 = 18$).

RESULTS

In this as well as the former study (6), the animals receiving triiodothyronine generally developed clinical evidence of hypermetabolism, e.g. hyperthermia, jumpiness, and a tendency to diarrhea within 24 hours after the first injection.

Table 2 lists the main effects and interactions of the three hormones and their combinations as regards a change in post-prandial blood sugar. Every treatment has produced an obvious elevation, but only in the case of the three main effects are these results statistically significant at the 1% level; none of the interactions achieve significance at the 5% level and hence the elevations in blood sugar following combined treatment must represent the additive effect of the individual hormones and not any synergism between them.

Table 3 lists the results in terms of glycosuria. While the main effect of both cortisone and growth hormone is a glycosuria significant at the 1% level, that of triiodothyronine is barely significant at the 5% level. None of the interactions achieve significance. The data are analyzed following a logarithmic transformation of the actual values, since total glycosuria is related to both the height and duration of any hyperglycemia above the renal threshold; it would be expected to increase geometrically as the hyperglycemia increases, and is therefore an exponential function. Further-

TABLE 2. MAIN EFFECTS AND INTERACTIONS OF THE TREATMENTS (TABLE 1) AS REGARDS NET INCREASE IN BLOOD SUGAR (IN MG.%)

Series	Treatment								Total
	1	2	3	4	5	6	7	8	
1	−7	33	26	75	4	51	133	64	379
2	12	8	59	40	34	63	70	129	415
3	−5	47	11	47	40	69	87	174	470
4	−3	24	19	54	27	64	69	73	327
5	−6	28	43	56	32	82	31	64	330
6	2	12	14	24	50	40	62	89	293
7	13	31	34	164	44	37	63	192	578
8	−4	−14	−1	76	32	45	50	46	230
9	−8	−8	1	17	93	65	55	151	366
Total	−6	161	206	553	356	516	620	982	3388
Mean	−.67	17.89	22.89	61.44	39.55	57.33	68.89	109.11	

Adjustment for mean $= (3388)^2 \div 72 = 159,424$
Treatment sum of squares $= 235,102 - 159,424 = 75,678$
Total sum of squares $= 292,830 - 159,424 = 133,406$
Residual sum of squares $= 133,406 - 75,678 = 57,728$
Mean error variance $= 57,728 \div 64 = 902.00$
Standard error $= \sqrt{902 \div 18} = \sqrt{50.1111} = 7.08$

		t	P
Main effect Cort.	$= \frac{1}{4}(245.77 - 130.66) = 28.77$	4.0635	<0.01
Main effect G.H.	$= \frac{1}{4}(262.33 - 114.10) = 37.06$	4.5866	<0.01
Main effect T3	$= \frac{1}{4}(274.88 - 101.55) = 43.33$	6.1200	<0.01
Interaction Cort. + G.H.	$= \frac{1}{4}(209.43 - 167.00) = 10.61$	1.4985	N.S.
Interaction Cort. + T3	$= \frac{1}{4}(188.66 - 187.77) = .22$	.0310	N.S.
Interaction G.H. + T3	$= \frac{1}{4}(195.22 - 181.21) = 3.50$	.4943	N.S.
Interaction Cort. + G.H. + T3	$= \frac{1}{4}(189.44 - 186.99) = .61$	.0861	N.S.

more this transformation enables the values to assume a more normal distribution. Both of these arguments have been considered adequate justification for the logarithmic transformation (11).

Table 4 lists the effects on nitrogen balance. It appears that growth hormone not only promotes nitrogen retention when given alone, but in combination with cortisone or triiodothyronine is also able to reverse the nitrogen wastage resulting from either of the latter hormones. However, with the triple combination the nitrogen balance is again negative. Statistical analysis reveals the main effects of growth hormone to be nitrogen retention, and of cortisone and triiodothyronine to be nitrogen wastage; in all cases $P = <0.01$. None of the interactions achieve statistical significance.

The effects on change in body weight are listed in Table 5 and resemble in every particular the effects on nitrogen balance.

DISCUSSION

The experimental design and analysis chosen for this study seem reasonable because they (1) are more efficient in that the treatments are evaluated with the same precision by means of only a quarter of the number of

TABLE 3. MAIN EFFECTS AND INTERACTIONS OF THE TREATMENTS (TABLE 1) AS REGARDS THE LOGARITHM OF THE NET GLYCOSURIA (IN MG./48 HRS.)

Series	Treatment								Total
	1	2	3	4	5	6	7	8	
1	1.748	2.875		3.461	2.566	2.728	3.371	3.366	20.115
2			2.853	3.344		2.812	2.634	3.396	15.039
3		2.892	2.447	3.447		2.152	2.810	3.473	17.221
4		2.246		3.096		2.745	3.155	3.041	14.283
5	1.945		2.576	3.224	1.973			2.845	12.563
6					2.158	2.265		3.328	7.751
7		2.384		3.653		2.009		3.545	11.591
8		1.602		3.193			2.447	2.250	9.492
9				1.017		2.362	3.088	3.331	9.798
Total	3.693	11.999	7.876	24.435	6.697	17.073	17.505	28.575	117.853
Mean	.410	1.333	.895	2.915	.744	1.897	1.945	3.175	

Adjustment for mean $= (117.853)^2 \div 72 = 192.907356$
Treatment sum of squares $= 252.889737 - 192.907356 = 59.982381$
Total sum of squares $= 338.258401 - 192.907356 = 145.351045$
Residual sum of squares $= 145.351045 - 59.982381 = 85.368664$
Mean error variance $= 85.368664 \div 64 = 1.333885$
Standard error $= \sqrt{1.333885} \div 18 = \sqrt{.074104} = .272$

		t	P
Main effect Cort.	$= \frac{1}{4}(9.320 - 3.994) = 1.332$	4.8971	<0.01
Main effect G.H.	$= \frac{1}{4}(8.930 - 4.384) = 1.137$	4.1801	<0.01
Main effect T3	$= \frac{1}{4}(7.761 - 5.553) = .552$	2.0294	<0.05
Interaction Cort. + G.H.	$= \frac{1}{4}(7.224 - 6.070) = .289$	1.0625	N.S.
Interaction Cort. + T3	$= \frac{1}{4}(6.377 - 6.937) = -.140$	−.5147	N.S.
Interaction G.H. + T3	$= \frac{1}{4}(6.863 - 6.451) = .103$	.3787	N.S.
Interaction Cort. + G.H. + T3	$= \frac{1}{4}(6.147 - 7.167) = -.255$	−.9375	N.S.

observations that would otherwise be necessary, (2) permit a statistical appraisal of the interactions as well as the main effects, and (3) have the theoretical advantage that measuring the treatment effects under a variety of conditions supplies a broader inductive basis for the conclusions. A fourth advantage, that this design allows for variation in animals or treatments between one replication and the next, unfortunately does not apply to his particular experiment for the reason previously given.

The results confirm previously established facts about the effects of adrenocortical, thyroid, and growth hormones on protein and carbohydrate metabolism. Both cortisone and triiodothyronine cause a hyperglycemia and glycosuria with negative nitrogen balance and weight loss. Growth hormone causes hyperglycemia and glycosuria in the face of nitrogen retention and accelerated weight gain.

While the present study was a progress, another experiment revealed growth hormone in the same dosage to be markedly diabetogenic in rats receiving excessive amounts of this same high carbohydrate diet; this effect occurred only when the rats received 44 ml. per day or more (1). None of the rats receiving growth hormone (alone or in combination) in the present study received this volume of diet at any time, so apparently

TABLE 4. MAIN EFFECTS AND INTERACTIONS OF THE TREATMENTS (TABLE 1) AS REGARDS NET NITROGEN BALANCE (IN MG. 48 HRS.)

Series	Treatment								Total
	1	2	3	4	5	6	7	8	
1	−24	−335	−164	−3	+201	−109	+3	−108	−539
2	−54	+167	+165	+144	−154	−309	+18	−23	−46
3	−39	−131	+15	−82	−34	−236	−26	+121	−412
4	−39	+223	+76	−142	−80	−314	−28	−166	−470
5	−56	−167	+179	−28	−178	−212	+15	−31	−478
6	−66	−114	+150	+219	−105	−180	−29	−108	−233
7	+30	−156	+146	+176	−54	−327	+126	−214	−273
8	+124	−155	+108	+93	−23	−161	+63	−171	−122
9	+50	+36	+136	+101	−252	−229	−42	+68	−132
Total	−74	−632	+811	+478	−679	−2077	+100	−632	−2705
Mean	−8.22	−70.22	+90.11	+53.22	−75.44	−230.77	+11.11	−70.22	

Adjustment for mean $= (-2705)^2 \div 72 = 101{,}625.34$
Treatment sum of squares $= 719{,}499.88 - 101{,}625.34 = 617{,}874.54$
Total sum of squares $= 1{,}519{,}455 - 101{,}625.34 = 1{,}417{,}829.66$
Residual sum of squares $= 1{,}417{,}829.66 - 617{,}874.54 = 799{,}955.12$
Mean error variance $= 799{,}955.12 \div 64 = 12{,}499.29$
Standard error $= \sqrt{12{,}499.29 \div 18} = \sqrt{694.40} = 26.35$

			t	P
Main effect Cort.	$= \frac{1}{4}(-317.99 - 17.56)$	$= -83.88$	−3.1833	<0.01
Main effect G.H.	$= \frac{1}{4}(84.22 - (-384.65))$	$= 117.22$	4.4485	<0.01
Main effect T3	$= \frac{1}{4}(-365.32 - 64.89)$	$= -107.55$	−4.0815	<0.01
Interaction Cort. + G.H.	$= \frac{1}{4}(-100.66 - (-199.77))$	$= 24.78$	.9404	N.S.
Interaction Cort. + T3	$= \frac{1}{4}(-219.10 - (-81.33))$	$= -34.44$	−1.3070	N.S.
Interaction G.H. + T3	$= \frac{1}{4}(-137.55 - (-162.88))$	$= 6.33$	.2402	N.S.
Interaction Cort. + G.H. + T3	$= \frac{1}{4}(-125.77 - (-174.66))$	$= 12.22$	.4637	N.S.

the carbohydrate load need not be as large as was previously supposed. Again the statistically significant results reported here point up the value of this experimental design in revealing differences that would otherwise go undetected.

In another study of the post-prandial carbohydrate tolerance of the triiodothyronine-treated rat, the shape and dimensions of the diabetic curve did not differ from those obtained during cortisone and growth hormone treatment (12). This makes it unlikely that the triiodothyronine-induced hyperglycemia was due to accelerated intestinal absorption of carbohydrate. However, in the present study the main effect of triiodothyronine on hyperglycemia was more pronounced than that of either cortisone or growth hormones, whereas its main effect on glycosuria was barely significant, in marked contrast to the effects of the other two agents. These discrepancies cannot be explained at the present time.

None of the interactions studied attained significant proportions in any of the parameters studied, leaving the inference that the result of any combination of these hormones was the algebraic sum of their independent effects. Thus the effect of every combination was to increase the hyperglycemia and glycosuria, the most striking diabetes occurring with the triple

combination. On the other hand the combination of growth hormone with either of the other two appeared to restore nitrogen balance and change in weight almost to baseline levels. These results are compatible with the idea that both cortisone and triiodothyronine administration can exagerate the diabetes resulting from concurrent growth hormone administration without necessarily resulting in a negative nitrogen balance in the whole animal.

The effects of triiodothyronine in this study resembled those of cortisone. However, no significant negative interaction between these two substances was observed, which might be interpreted to mean that there is no obvious competition for a final metabolic pathway. Further speculation along these lines is unjustified.

The failure to demonstrate any significant interactions was a little surprising, but its implications are meaningful only within the limits of the experimental stituation. One such limit is the size of the experiment: by calculation, a significant interaction between growth hormone and cortisone is theoretically attainable if the number of animals were doubled. Even then, the values would be minor compared to the main effects. Another limit is the duration of the experiment. Greenberg and Aterman,

TABLE 5. MAIN EFFECTS AND INTERACTIONS OF THE TREATMENTS (TABLE 1) AS REGARDS NET CHANGE IN WEIGHT (IN GM./24 HRS.)

Series	Treatment								Total
	1	2	3	4	5	6	7	8	
1	+0.3	− 6.8	+ 1.0	−2.2	−11.3	−10.3	0.0	− 0.8	− 30.1
2	0.0	− 4.0	+ 3.8	−3.5	− 5.0	− 9.8	− 1.5	− 4.5	− 24.5
3	+1.3	− 5.3	+ 4.1	−3.0	− 4.3	− 8.8	− 1.5	− 4.3	− 21.8
4	−1.5	− 3.8	+ 6.3	+4.5	− 6.8	− 8.8	− 0.7	− 6.8	− 17.6
5	−2.5	− 4.8	+ 2.3	+1.8	− 4.3	− 6.5	− 2.8	− 5.5	− 22.3
6	−1.2	− 0.8	+ 7.3	+2.3	− 3.8	− 5.0	− 2.0	− 2.0	− 5.2
7	+3.0	− 3.3	+10.5	+1.3	− 1.5	− 8.3	+ 1.3	− 3.0	0.0
8	+1.5	− 2.8	+ 3.5	+2.8	− 2.8	− 9.5	− 2.2	− 7.2	− 16.7
9	+1.8	− 2.3	+ 6.3	−0.5	− 4.0	− 8.7	− 3.0	− 5.3	− 15.7
Total	+2.7	−33.9	+45.1	+3.5	−43.8	−75.7	−12.4	−39.4	−153.9
Mean	+0.3	− 3.8	+ 5.0	+0.4	− 4.9	− 8.4	− 1.4	− 4.4	

Adjustment for mean $= (-153.9)^2 \div 72 = 328.96$
Treatment sum of squares $= 1395.31 - 328.96 = 1066.35$
Total sum of squares $= 1712.73 - 328.96 = 1388.77$
Residual sum of squares $= 1388.77 - 1066.35 = 322.42$
Mean error variance $= 322.42 \div 64 = 5.03$
Standard error $= \sqrt{5.03 \div 18} = \sqrt{.27} = .52$

		t	P
Main effect Cort.	$= \frac{1}{4}(-16.2 - (-1.0)) = -3.80$	−7.30	<0.01
Main effect G.H.	$= \frac{1}{4}(-0.4 - (-16.8)) = 4.10$	7.88	<0.01
Main effect T3	$= \frac{1}{4}(-19.1 - 1.9)) = -5.25$	−10.09	<0.01
Interaction Cort. + G.H.	$= \frac{1}{4}(-8.6 - (-8.6)) = 0.00$		N.S.
Interaction Cort. + T3	$= \frac{1}{4}(-7.5 - (-9.7)) = 0.55$	1.05	N.S.
Interaction G.H. + T3	$= \frac{1}{4}(-9.3 - (-7.9)) = 0.35$	−0.67	N.S.
Interaction Cort. + G.H. + T3	$= \frac{1}{4}(-8.1 - (-9.1)) = 0.25$	0.48	N.S.

using roughly comparable doses of cortisone and powdered thyroid in intact weanling rats, demonstrated antagonistic effects on growth after four weeks of treatment, although this was inapparent after one week (13).

A third consideration arises from the fact that each of the hormones was administered in an unphysiologically large dose in an attempt to produce a prompt metabolic derangement. Homeostatic mechanisms would be expected to resist these influences vigorously and might obscure a synergistic action which would be apparent in the reverse situation, i.e. replacement therapy in the hypophysectomized organism. In hypophysectomized dogs de Bodo has found that a small dose of cortisone alone abolishes insulin sensitivity and restores glucose tolerance to normal, whereas no dose of growth hormone alone will abolish insulin sensitivity without resulting in a somewhat diabetic glucose tolerance curve. Yet combined treatment with these two hormones in these doses produces normal insulin sensitivity and glucose tolerance as well (14). It seems highly probable that a statistically significant negative interaction between these two hormones must occur in this situation. Furthermore, Simpson *et al.* (15) feel that a synergism between growth hormone and thyroxine occurs in the growth response of hypophysectomized rats to combined treatment.

Finally, studies on the whole animal measure isolated biochemical effects in an admittedly crude and indirect fashion; true "synergism" and "competition" might best be reserved to describe phenomena occurring at the cellular or subcellular level.

Regardless of methodology, this experimental design clearly permits a more thorough extraction of information from the data. It will undoubtedly be of great value in any study which attempts to separate truly synergistic effects of treatment combinations from those which are merely additive.

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NOTES AND COMMENTS

DISSOLUTION OF EXPERIMENTAL VESICAL CALCULI BY HYPOPHYSECTOMY IN RATS[1]

ABSTRACT

Groups of rats bearing experimentally induced calculi in their urinary bladders were subjected to hypophysectomy and sham-hypophysectomy. Hypophysectomy led to an average reduction of 79% of the stone weight in 32 of 33 animals. In 34 of 40 sham-hypophysectomized rats, an average gain in stone weight of 85% was observed. Since no significant differences between the two groups, with the exception of the changes incident to the hypophysectomy, were observed, the effects were attributed to hypophysectomy.

In previous experiments, we have discovered that the glands of internal secretion have a pronounced influence on the rate of formation and the incidence of occurrence of calculi formed in the urinary bladders of rats in response to aseptically implanted magnesium metal niduses (1, 2, 3). Androgens stimulate, and estrogens inhibit calculogenesis. Hypophysectomy prevents stone formation. The present experiments show that hypophysectomy has considerable therapeutic merit in dissolution of established calculi in rats.

MATERIALS AND METHODS

Long-Evans male rats, weighing 120–175 gm., were aseptically implanted with 15 mg. cylindrical, C.P. magnesium ribbon niduses, by the method of Vermeulen and co-workers (4), slightly modified. All operations were done under either anesthesia.

Bladders were re-opened four weeks after implantation of the magnesium ribbon. Primary stones were blotted and weighed immediately and replaced into the urinary bladder after all secondary, or daughter stones were removed. The urinary pH was estimated with short range test paper, and body weights were recorded. Hypophysectomy and sham-hypophysectomy were performed three days later.[2] The controls remained in our animal laboratory Each rat served as his own control, having demonstrated an ability to form stones.

All rats were placed in a warm room with a constant temperature of 80–82° F after hypophysectomy. The animals were fed Purina Laboratory Chow and given water *adlibitum*.

Twenty-four hour fluid intake was recorded daily for one week prior to removal of the stones. After four weeks, the bladders were re-opened; urinary pH, primary stone weights and final body weights were determined. Statistical analysis was made by the method of Fisher (5), and probability was taken from his table.

RESULTS

All rats developed calculi around the magnesium ribbon niduses four weeks after implantation. At this time, the average urinary pH was 6.8 in all groups. During the treatment period, the hypophysectomized rats and the controls had approximately equal 24-hour fluid intake, averaging 56.8 cc. for the controls, 65.9 cc. for sham-operated and 57.0 cc. for hypophysectomized animals. Five animals did not survive hypophysectomy. The remaining animals of all groups were in good health and none were infected.

Table 1 shows that there was a marked decrease in stone weights in the hypophysectomized group of animals. There was only one in the group of 33 animals whose

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[2] By Diablo Animal Laboratories, Berkeley, California. Animals were transported by air.

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND SHAM HYPOPHYSECTOMY ON GROWTH OF PREFORMED EXPERIMENTAL VESICAL CALCULI. EACH TREATMENT GROUP IS DIVIDED INTO TWO GROUPS, ONE IN WHICH THE STONE INCREASED IN SIZE DURING THE TREATMENT PERIOD, THE OTHER IN WHICH THE STONE DECREASED IN SIZE. THESE CHANGES ARE DESIGNATED BY PLUS AND MINUS SIGNS

Group	No. of rats	% animals ±stone wt.	Average % ±stone wt.	Average ±stone wt. in mg.	Average urinary pH	Average stone wt. in mg.	
						Before treatment	After treatment
Hypophysectomy	33	+ 3.0 −97.0	+58.5 −79.0	+32.5 −70.4	7.0	+ 55.6 − 86.4	+ 88.0 − 16.0
Sham-hypophysectomy	40	+85.0 −14.4	+75.5 −20.7	+53.2 −25.8	6.8	+ 82.0 −111.4	+135.1 − 85.7
Untreated controls	37	+78.4 −21.6	+91.8 −20.5	+44.6 −17.7	6.8	+ 56.0 − 89.5	+101.2 − 71.9

stone weight increased. It gained 32.5 mg. Careful dissection of the pituitary fossa failed to disclose residual pituitary tissue. The failure of growth and the hypoplastic gonads and generative organs were further evidence suggesting hypophysectomy was complete. Eighty-five per cent of sham-hypophysectomized animals and 78.4% of the controls showed increased stone weights. The average urinary pH in the hypophysectomized group was 7.0, and that of the other two groups was 6.8. Hypophysectomized animals lost an average of 16.8% of initial body weight in 4 weeks. Sham-hypophysectomized rats, except one, gained an average of 21.5% .The controls gained an average of 29% of initial body weight. Statistical analysis showed that all the differences between the hypophysectomy group and the two control groups were highly significant ($P<.001$). The control group had significantly larger ($P<0.02$) calculi than the sham-hypophysectomy group.

DISCUSSION

Hypophysectomy greatly reduces steroid hormone production. It was shown in previous studies (1, 2, 3) in rats that estrogen antagonizes, and androgen favors experimental calculus formation in Long-Evans rats. Experimental hypophysectomy reduced urinary excretion of calcium, magnesium, and phosphate. Replacement therapy with luteotrophin and testosterone increased urinary magnesium excretion; gonadotrophins increased calcium in the urine; gonadotrophins and ACTH increased both calcium and magnesium, and growth hormone increased urinary phosphate excretion. The increased excretion of these ions favored the formation of urinary concrements.

Hypophysectomy prior to implantation of the magnesium nidus in the urinary bladder in rats abolished the tendency to stone formation, and hypophysectomy after the calculus formed led to a marked reduction in the size of the stone, including the magnesium nidus implanted. The stress of sham-hypophysectomy led to less increase in size of calculi. These results are not in disagreement with those of Marshall and co-workers (6), who found that repeated stress increased stone size. In our experiments there was only one stress stimulus. The increase in urinary pH in the hypophysectomized group was probably not significant or sufficiently marked to produce larger stones.

DONALD F. MCDONALD AND MARIA O. ORALLO

Division of Urology
Department of Surgery
University of Washington School of Medicine
Seattle, Washington

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